



UNIVERSIDADE NOVA DE LISBOA

**THE INHIBITORY ACTIVITY OF SYNTHETIC COMPOUNDS AND IONS
AGAINST TRANSPORTERS OF MULTI-DRUG RESISTANT BACTERIA**

LISA SANTOS MACHADO

**DISSERTATION TO OBTAIN A MASTER'S DEGREE IN MEDICAL
MICROBIOLOGY**

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ABSTRACT

Sixty-three hydantoin derivatives were evaluated for their modulating effects on efflux pump (EP) activity of *Salmonella* NCTC 13349 utilizing a semi-automatic fluorometric method. None of the compounds presented antibacterial activities at concentrations as high as 240 mg/L. Among all compounds, SZ-7 showed possible efflux modulating activity in the presence of glucose, indicative of a potential EP inhibitor. To verify its potential effects, ciprofloxacin-resistant *Salmonella* strains, induced to high level resistance with over-expressing EPs, were exposed to SZ-7. This derivative affected the susceptibility of the ciprofloxacin-resistant strains.

Since the 63 compounds studied had very low inhibitory/accumulative effects, even though their known for being efficient in modulating ion-driven eukaryotic EPs, we questioned whether ions had a leading role in regulating bacterial EPs, influencing the effectiveness of new compounds. For this study we used *Escherichia coli* AG100 as a model, due to the extensive knowledge on its EPs structure and activity.

Owing the importance of calcium ions (Ca^{2+}) for membrane transport channels and activity of ATPases, the role of Ca^{2+} was investigated. From previous results we concluded that at pH 5 efflux is independent of metabolic energy; however, at pH 8 it is entirely dependent of metabolic energy and the Ca^{2+} ions are essential to maintain the activity of bacterial ATPases.

Accumulation and efflux of ethidium bromide (EtBr) by *E. coli* AG100 was determined in the presence and absence of Ca^{2+} , chlorpromazine (inhibitor of Ca^{2+} -binding to proteins), and ethylenediaminetetraacetic acid (Ca^{2+} chelator). Accumulation of EtBr increased at pH 8; however Ca^{2+} reversed these effects providing information as to the importance of this ion in the regulation of bacterial EP systems.

Overall this work puts in evidence that many biochemical and bioenergetic aspects related to the strains physiology need to be taken into consideration in bacterial drug resistance mediated by EPs.

RESUMO

Sessenta e três derivados de hidantoína foram utilizados para avaliar possíveis efeitos de modulação na actividade das bombas de efluxo (BE) na *Salmonella* NCTC 13349 utilizando um método fluorimétrico semi-automático. Nenhum dos compostos apresentaram actividade anti-bacteriana até concentrações de 240 mg/L. Entre todos os compostos, SZ-7 demonstrou possuir propriedades de modulação de efluxo na presença de glucose. Para testar esta actividade, estirpes de *Salmonella* resistentes à ciprofloxacina, induzidas a elevados níveis de resistência com sobre-expressão de BE, foram expostas ao SZ-7. Este derivado afectou a susceptibilidade das estirpes à ciprofloxacina.

Uma vez que os 63 compostos estudados apresentaram pouco efeito inibitório/cumulativo, apesar de serem conhecidos pelos seus efeitos moduladores de BE-dependentes de iões em eucariotas, foi questionado o papel dos iões na regulação de BE bacterianas, que poderão influenciar a eficácia de novos compostos. Para este estudo, utilizamos a *Escherichia coli* AG100 como modelo, devido ao extenso conhecimento no que respeita a estrutura e actividade das BE.

Devido à importância de iões de cálcio (Ca^{2+}) nos canais de transporte membranar e na actividade de ATPases, a sua actividade na modulação do efluxo foi investigada. De resultados anteriormente obtidos concluiu-se que a pH 5 o efluxo é independente de energia metabólica; contudo, a pH 8 é absolutamente dependente, sendo que o Ca^{2+} é indispensável para manter a actividade das ATPases bacterianas.

A acumulação/efluxo de EtBr pela *E. coli* AG100 foi determinada na presença/ausência de Ca^{2+} , clorpromazina (inibidor de ligação de Ca^{2+} a proteínas), e ácido etilenodiamino tetra-acético (quelante de Ca^{2+}). Acumulação/efluxo aumentou a pH 8, contudo o Ca^{2+} reverte estes efeitos evidenciando a sua importância no funcionamento das BE bacterianas.

Em resumo este trabalho colocou em evidência que muitos aspectos bioquímicos e bioenergéticos devem ser tomados em consideração no estudo da resistência bacteriana mediada por BE.

TABLE OF CONTENTS

PUBLICATIONS	i
ACKNOWLEDGEMENTS	ii
ABSTRACT.....	iv
RESUMO.....	v
TABLE OF CONTENTS	vi
FIGURE INDEX.....	xi
TABLE INDEX	xiii
LIST OF ABBREVIATIONS	xv
I. INTRODUCTION	1
1. Enterobacteriaceae.....	1
1.2. Virulence Factors.....	1
1.3. Epidemiology	2
1.4. <i>Salmonella enterica</i> and <i>Escherichia coli</i>	3
1.4.1. <i>Escherichia coli</i>	3
1.4.2. <i>Salmonella enterica</i>	4
2. Mechanisms of Action and Resistance to Antibiotics	6
2.1. Efflux Pumps.....	7
2.1.1. Classification of Multidrug Efflux Pumps	8
2.1.1.1. “ATP-binding cassette” (ABC) superfamily.....	9
2.1.1.2. “Major Facilitator” superfamily (MFS)	10
2.1.1.3. “Multidrug and Toxic Compound Extrusion” (MATE) family	10
2.1.1.4. “Small Multidrug Resistance” (SMR) family	11

2.1.1.5. “Resistance Nodulation Division” (RND) superfamily	11
2.1.2. Efflux systems in <i>E. coli</i> and <i>Salmonella enterica</i>	15
2.1.3. Efflux pump systems: Physiological Importance	19
2.1.3.1. Importance of Calcium Regulation	20
3. MDR Efflux Inhibitors	21
3.1. Calcium channel inhibitors	23
3.1.1. Phenothiazines	23
3.1.1.1. Chlorpromazine	24
3.2. Novel Compounds	25
3.2.1. Hydantoin compounds	26
4. Detection methods of accumulation and efflux in bacteria	28
4.1. Semi-automatic fluorometric method to monitor ethidium bromide transport through bacterial membranes	28
II. STUDY OBJECTIVES	31
III. MATERIAL AND METHODS	33
1. Assessment of the role of synthesized hydantoin derivatives in various <i>Salmonella enterica</i> strains	33
1.1. Materials	33
1.1.1. Bacterial Strain	33
1.1.2. Culture media, compounds and solutions	33
1.2. Methods	35
1.2.1. Cultivation of bacterial strains <i>Salmonella</i> NCTC 13349 and ciprofloxacin-resistant <i>Salmonella</i> mutant’s 104-CIP and 5408-CIP for MIC determinations	35
1.2.2. Cultivation of bacterial strains for the fluorometric accumulation assays	36

1.2.3. Determination of the MIC for <i>Salmonella enterica</i> NCTC 13349 reference strain in the presence of hydantoin derivatives, EtBr, TZ and DMSO	36
1.2.4. Fluorometric assay performed by the Real-Time Thermal Cyclor Rotor-Gene TM 3000	38
1.2.4.1. EtBr accumulation assay to assess the effect of hydantoin derivatives in <i>Salmonella enterica</i> NCTC 13349	39
1.2.4.2. Auto-fluorescence assay.....	42
1.2.5. Determination of MIC for two ciprofloxacin-resistant <i>Salmonella</i> mutant strains in the presence of hydantoin compound SZ-7, ciprofloxacin and TZ.....	42
1.2.6. Fluorometric assay to assess the effect of SZ-7 on the accumulation of EtBr in two ciprofloxacin-resistant <i>Salmonella</i> mutant strains	44
1.2.6.1. Accumulation protocol of EP substrate EtBr	44
2. Assessment of the role of calcium in <i>E. coli</i> reference strain, in the presence of chlorpromazine and ethylenediaminetetraacetic acid.....	46
2.1. Materials	46
2.1.1. Bacterial Strain	46
2.1.2. Culture media, compounds and solutions	46
2.2. Methods.....	47
2.2.1. Cultivation of bacterial strain <i>E. coli</i> AG100 for MIC determinations	47
2.2.2. Cultivation of bacterial strain <i>E. coli</i> AG100 for the fluorometric accumulation and efflux assays	48
2.2.3. Determination of MIC for <i>E. coli</i> AG100 reference strain in the presence of CPZ, EDTA and EtBr.....	48
2.2.4. Fluorometric assay performed by the Real-Time Thermal Cyclor Rotor-Gene TM 3000	49
2.2.4.1. Accumulation protocol of EP substrate EtBr	49
2.2.4.2. Efflux protocol of EP substrate EtBr	52

IV. RESULTS	53
1. Assessment of the role of newly synthesized hydantoin derivates in various <i>Salmonella enterica</i> strains.....	53
1.1. MIC values obtained for the hydantoin compounds, EtBr and TZ by <i>Salmonella enterica</i> NCTC 13349 reference strain.....	53
1.2. Accumulation of EtBr in the presence of various hydantoin derivates in <i>Salmonella enterica</i> NCTC 13349 by the fluorometric assay	56
1.2.1. Auto-fluorescence assays obtained by the hydantoin derivatives	57
1.2.2. Relative final fluorescence values obtained by the hydantoin derivatives	59
1.2.2.1. Fluorometric assay obtained for hydantoin derivative SZ-7	60
1.3. MIC values obtained for <i>Salmonella</i> mutants 104-CIP and 5408-CIP in the presence of ciprofloxacin, hydantoin compound SZ-7, TZ, and EtBr	61
1.4. Accumulation of EtBr in the presence of hydantoin derivate SZ-7 in <i>Salmonella</i> mutants 104-CIP and 5408-CIP by the fluorometric assay	62
1.4.1. Effect of hydantoin derivative SZ-7 on accumulation of EtBr in <i>Salmonella</i> mutants 104-CIP and 5408-CIP	63
1.4.2. Effect of TZ on accumulation of EtBr in <i>Salmonella</i> mutants 104-CIP and 5408-CIP.....	64
2. Assessment of the role of calcium in <i>E. coli</i> AG100, in the presence of CPZ and EDTA	66
2.1. MIC values obtained for <i>E. coli</i> AG100 in the presence of CPZ, EDTA and EtBr.....	66
2.2. Accumulation of EtBr in the presence of CPZ and EDTA to assess the role of calcium in <i>E. coli</i> AG100 strain by the fluorometric assay	67
2.2.1. Effect of CPZ on accumulation of EtBr and modulation of that effect by Ca ²⁺ ions	67
2.2.1.1. Effect of CPZ at varying concentrations on the accumulation of EtBr	67
2.2.1.2. Ca ²⁺ modulation of the effects of CPZ on accumulation of EtBr.....	68

2.2.2. Effect of EDTA on accumulation of EtBr and modulation of that effect by Ca^{2+} ions	69
2.2.2.1. Effect of EDTA at varying concentrations on the accumulation of EtBr	69
2.2.2.2. Ca^{2+} modulation of the effects of EDTA on accumulation of EtBr	69
2.2.3. Effects of CPZ with and without Ca^{++} on efflux of EtBr.....	70
 V. DISCUSSION AND CONCLUSION	 72
Future Perspectives	80
 VI. BIBLIOGRAPHIC REFERENCES	 81

FIGURE INDEX

Figure 1 – Diagrammatic comparison of the five families of bacterial EPs.....	9
Figure 2 – Schematic representation of the tripartite efflux system AcrAB-TolC	12
Figure 3 – Structural model for multidrug transporters of the RND family	13
Figure 4 – Reaction scheme for the enzymatic cleavage of D, L-5-monosubstituted hydantoin derivatives to the corresponding D- or L-amino acids.....	28
Figure 5 – Accumulation of substrate EtBr in presence and absence of a potential inhibitor of EPs	30
Figure 6 – Schematic representation of the fluorescence values selected to calculate the RFF value.....	41
Figure 7 – Accumulation of EtBr at varying concentrations by <i>Salmonella enterica</i> NCTC 13349.....	55
Figure 8 – Accumulation of EtBr by <i>Salmonella enterica</i> NCTC 13349 in the presence of varying concentrations of TZ.....	57
Figure 9 – Auto-fluorescence assay performed for hydantoin compound SZ-2.....	58
Figure 10 – Accumulation of EtBr by <i>Salmonella enterica</i> NCTC 13349 in the presence of varying concentrations of SZ-7.....	60
Figure 11 – Accumulation of EtBr by <i>Salmonella</i> mutant's 104-CIP and 5408-CIP in the presence of varying concentrations of SZ-7.....	63
Figure 12 – Accumulation of EtBr by <i>Salmonella</i> mutant's 104-CIP and 5408-CIP in the presence of varying concentrations of TZ.....	64
Figure 13 – Effect of chlorpromazine (CPZ) on accumulation of EtBr	68
Figure 14 – Modulation of the effect of CPZ by calcium ions (Ca^{2+}).....	68

Figure 15 – Effect of EDTA on the accumulation of EtBr.....	69
Figure 16 – Modulation of the effect of 5 and 10 mM of EDTA by Ca ²⁺ ions.....	70
Figure 17 – Effects of chlorpromazine (CPZ) with and without Ca ²⁺ on efflux of EtBr by <i>E. coli</i> AG100 strain	71

TABLE INDEX

Table I – Bacterial EPs involved in antibiotic resistance by <i>E. coli</i> and respective characteristics.....	16
Table II – Composition of culture media	33
Table III – Composition and preparation method of the solutions used throughout the assays.....	34
Table IV – Composition and preparation method of antibiotic ciprofloxacin, EPI TZ and hydantoin derivatives	34
Table V – Composition and preparation method of substrate EtBr used by bacterial EPs	35
Table VI – Range of concentrations used in determining the MIC for hydantoin compounds, for EtBr and for TZ. Evaluation of the effect of 10% of DMSO	37
Table VII – Range of concentrations used for the MIC determinations of ciprofloxacin, hydantoin derivative SZ-7 and phenothiazine TZ.....	43
Table VIII – Composition of culture media	46
Table IX – Composition and preparation method of the solutions	46
Table X – Composition and preparation method of ATPase inhibitor CPZ and Ca ²⁺ chelator EDTA	47
Table XI – Range of concentrations used for the MIC determination of CPZ, EDTA and EtBr.....	48
Table XII - MIC values obtained by <i>Salmonella enterica</i> NCTC 13349 for substrate EtBr and for TZ. Also, the result obtained from the assay used to evidence bacterial growth in the presence of DMSO at 10%	53
Table XIII – MIC values obtained for all hydantoin derivatives under study by <i>Salmonella enterica</i> NCTC 13349	54

Table XIV – Relative final fluorescence (RFF) based on accumulation of EtBr	59
Table XV – MIC values obtained for antibiotic ciprofloxacin, hydantoin compound SZ-7, phenothiazine TZ and substrate EtBr by <i>Salmonella enterica</i> 104-CIP and 5408-CIP	61
Table XVI – MIC values obtained by <i>E. coli</i> AG100 in the presence of EPI CPZ, Ca ²⁺ chelator EDTA and substrate EtBr.....	66
Table XVII – Hydantoin derivatives under study to evaluate their potential as EPIs ..	95

LIST OF ABBREVIATIONS

Å	Ångström unit
AA	Amino acid
ABC	Adenosine triphosphate (ATP)-Binding Cassette
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine triphosphatase
bp	Band-pass
Ca ²⁺	Calcium
[Ca ²⁺] _i	Intracellular free-Ca ²⁺ concentration
CaBP	Ca ²⁺ -binding protein
CaCl ₂	Calcium Chloride
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CO ₂	Carbon dioxide
CPZ	Chlorpromazine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EP	Efflux Pump

EPI	Efflux Pump Inhibitor
EtBr	Ethidium bromide
Fe ³⁺	Iron
g	Gram
g/mL	Grams per milliliter
Glu	Glucose
H ⁺	Hydron
HCl	Hydrochloric acid
hp filters	High-pass (hp) filters
IFN	Interferon
IL	Interleukin
IMP	Inner-membrane protein
K ⁺	Potassium
KAN	Kanamycin
KCN	Potassium cyanide
L	Litre
LA	Luria Bertani agar
LB	Luria Bertani
LPS	Lipopolysaccharides
M	Molar
MATE	Multi-Antibiotic Toxin Extrusion
MDR	Multidrug resistance

MFP	Membrane fusion protein
MFS	Major Facilitator Superfamily
mg	Milligram
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
Mg ²⁺	Magnesium
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
mL	Millilitre
mM	Millimolar
Na ⁺	Sodium
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCTC	National Collection of Type Cultures
nm	Nanometre
NSAIDs	Non-steroidal anti-inflammatory agents
°C	Celsius
OD	Optical density
OMP	Outer membrane protein
PAβN	Phenylalanyl arginyl-β-naphthylamide

PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
Pi	Inorganic phosphate
pKa	Acid dissociation constant
PMF	Proton motive force
PMQR	Plasmid-mediated quinolone resistance
RF	Relative fluorescence
RFF	Relative final fluorescence
RNA	Ribonucleic acid
RND	Resistance Nodulation Division
rpm	Revolutions per minute
SMR	Small Multidrug Resistance
spp.	Species
TET	Tetracycline
TMS	Transmembrane segments
TZ	Thioridazine
v	Volume
v/v	Volume fraction
Δ pH	Chemical proton gradient
$\Delta\Psi$	Electrical potential
μ m	Micrometre

I. INTRODUCTION

1. Enterobacteriaceae

Enterobacteriaceae are a large and diverse family of Gram-negative bacteria, members of which are both free-living (distributed on plants, in soil and water) and part of the indigenous flora of humans and animals (15, 36, 38). They colonize the intestinal tracts of humans (58) but are relatively uncommon as normal flora of other body sites (15).

They are among the largest bacteria, measuring 2 to 4 μm in length, with forms that range from large coccobacilli to elongated, filamentous rods (36, 58). They grow rapidly under aerobic or anaerobic conditions and are metabolically active, fermenting (rather than oxidizing) D-glucose and other sugars (15, 36), often with gas production (15). Most genera and species in this family include the following characteristics: do not form spores, are motile by peritrichous flagella or non-motile, are catalase positive and oxidase negative, reduce nitrates to nitrites (15, 36, 58), and have a 39 to 59% guanine-plus-cytosine content of DNA (15).

1.2. Virulence Factors

The cell envelope of Gram-negative bacteria consists of an outer cell wall, a periplasmic space and the plasma membrane (54). They are protected by a thick double-membrane (26), which is highly hydrophobic due to the presence of a lipopolysaccharides (LPS), providing these organisms with a substantial permeability barrier (41, 88, 99). They constitute efficient obstacles towards hydrophilic molecules (10), most of which can gain access to targets beyond the outer cell wall via conduits that begin at the surface of the cell and end at the plasma membrane (54). On the other hand, they are easily crossed by amphiphilic compounds, since these are able to diffuse through both the hydrophilic and the hydrophobic domains of the bilayer. Therefore, it is not surprising that mechanisms were devised to protect cells from the disordered invasion by amphiphilic molecules, many of which are endowed with biological activities that lead to harmful effects (10).

Another important factor relates to the ability of these organisms to spread in the bloodstream producing an endotoxic shock, a potentially lethal manifestation of infection (36, 38). Endotoxins are pharmacologically active LPS contained within the cell walls of Gram-negative species (19, 36). These polysaccharides are structured into three regions: (1) an external variable portion of carbohydrate exposed to the environment and the defense system of a potential host that determines the antigenic specificity called the O antigen (e.g., multiple serotypes of *Salmonella*) (19, 36, 38), (2) a polysaccharide center or core which is structurally similar among species and (3) a highly conserved lipid portion called lipid A (27, 38). Motile strains have protein peritrichous flagella, which extend well beyond the cell wall and are called the H antigen (36).

In addition to the LPS endotoxin common to all Gram-negative bacteria, some *Enterobacteriaceae* also produce protein exotoxins, which act on host cells by damaging membranes, inhibiting protein synthesis, or altering metabolic pathways, leading to cellular death or physiologic alteration (36).

1.3. Epidemiology

Most *Enterobacteriaceae* are primarily colonizers of the lower gastrointestinal tract of humans and animals (36), exposing it to harmful exogenous substances and functioning as a host defense/detoxification barrier (103). They can also be found in the female genital tract and as transient colonizers of the skin. *Enterobacteriaceae* are scant in the respiratory tract of healthy individuals; however, their numbers increase in hospitalized patients with chronic debilitating diseases (36).

E. coli is the most common species of *Enterobacteriaceae* found among the indigenous flora, followed by *Klebsiella*, *Proteus*, and *Enterobacter* species. *Salmonella* and *Shigella* species are not considered members of the normal flora, although carrier states can exist (36).

Statistically, several species of *Enterobacteriaceae* are very important causes of nosocomial infections, and account for nearly 50% of septicemia cases, more than 70% of urinary tract infections, and a significant percentage of intestinal infections (15).

They are the most frequently isolated bacteria from biological samples (38), often associated with abscesses, pneumonia, meningitis, septicemia, and infections of wounds, urinary tract, and the intestine (15).

Therefore it comes as no surprise that the Infectious Diseases Society of America has recognized Gram-negative pathogens as some of the most problematic bacterial challenges (16, 18, 40). Not only is the increase emergence of resistance in Gram-negative bacteria faster than in Gram-positive bacteria (21, 40), but also there are fewer new antibiotics active against Gram-negative bacteria and drug development programs seem insufficient to provide therapeutic cover in 10–20 years (40). This ineffectiveness is largely due to the excessive use of antibiotics in human therapy, as well as for farm animals, leading to a selection of pathogenic bacteria resistant to multiple drugs (68).

1.4. *Salmonella enterica* and *Escherichia coli*

An important property common to both species relates to the route of transmission (fecal-oral), which gives them the ability to survive in acidic environments (5, 43). With their passage into the small intestine, organisms will encounter a less acidic environment (pH 4 to 6) near neutral (5, 43), but one that includes the presence of fermentation end products (weak acids) produced by the normal intestinal flora. Although the pH of the intestinal contents is less acidic than that of the stomach, the presence of weak acids will increase acid stress to potentially lethal levels for enteric bacteria, such as *E. coli* and *Salmonella* spp. Nevertheless, these pathogens will survive gastrointestinal acidity and ultimately cause disease (43).

1.4.1. *Escherichia coli*

While recognized as a commensal organism, *E. coli* is by far the most common cause of urinary tract infections (36, 84). This organism shows marked tropism for different human epithelial cell types due to the presence of pili, which plays a key role in virulence (36) due to their capacity to adhere to host cells and to form biofilms (6). The P pili and type 1 pili are surface organelles attached to the outer membrane and frequently found on these uropathogenic isolates. Bacteria expressing P pili are most

commonly linked with infections in the upper urinary tract and kidney region causing pyelonephritis, while bacteria expressing type 1 pili are usually found in the lower urinary tract and bladder causing cystitis (6).

Enteropathogenic and enterotoxigenic *E. coli* are a common cause of diarrhea (36, 84) in developing countries and for travelers. If antimicrobial therapy is indicated, the same agents are often used as for the treatment of urinary tract infections, i.e., fluoroquinolone, trimoxazole or nitrofurantoin (84). In children and the immunocompromised, *E. coli* can cause more serious infections, associated with higher morbidity and mortality. For these patient groups, antimicrobial treatment with a broad-spectrum cephalosporin or a fluoroquinolone is required (84).

Regarding resistance in *E. coli*, wild-type strains are resistant to most lipophilic antibiotics, which includes penicillin G, oxacillin, cloxacillin, nafcillin, macrolides, novobiocin, linezolid, and fusidic acid (68, 69).

1.4.2. *Salmonella enterica*

Based on iso-enzymes, rRNA sequences, and DNA hybridization, microbial taxonomists consider almost all of the *Salmonella* that infect mammals and birds to belong to one species – *S. enterica*. There are six subspecies of *S. enterica* including *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (27). Within these six subspecies are over 2,000 serotypes of *Salmonella* (27, 90), as defined by the carbohydrate structures found in LPS and by flagella antigens. Nearly all the serotypes that cause disease in humans and domestic animals belong to subspecies *enterica*. Despite the fact that they are genetically closely related, there is enormous variation in the virulence and epidemiology of different serotypes of *S. enterica* (27).

Until the 1980s *S. enterica* serovar Typhimurium (*S. Typhimurium*) was the most common serovar isolated from humans worldwide. However, in the late 1980s *S. enterica* serovar Enteritidis emerged as the most common cause of human salmonellosis and soon became the most prevalent serovar in many countries worldwide (12, 71).

Salmonella is widespread in nature and can colonize or infect a wide variety of mammals, birds and reptiles (114), resulting in many different syndromes ranging from

colonization and chronic carriage to acute fatal disease (27). Salmonellosis designates the common food-borne infection caused by *Salmonella* spp in industrialized countries (61) that owes its large outbreaks to the ingestion of contaminated food of animal origin including eggs, meat, unpasteurized dairy products, seafood, fruits and vegetables (12, 35, 114).

Infection with *Salmonella enterica* serovar Typhi, which appears to be limited to human hosts, causes a spectrum of illness that includes diarrhea, a self-limited febrile illness, and most significantly, typhoid fever (29).

Nontyphoid *Salmonella* are also responsible for enteric fever, gastroenteritis and bacteremia in humans and a broad array of illnesses in animals, including enteritis and septicemia in livestock (17, 27). However, non-typhoid *Salmonella* strains can also cause a variety of life-threatening extra-intestinal infections (27), since the *spv* (*Salmonella* plasmid virulence) genes appear to promote the macrophage phase of the disease process, avoiding destruction by neutrophils and facilitating proliferation at these sites of infection (17). A number of host factors can predispose to extra-intestinal *Salmonella* infections, including AIDS, hemolytic anemias, and genetic defects that affect IL-12, IFN- γ , and the phagocyte NADPH oxidase, but even in the susceptible host, certain serotypes of *Salmonella* are more likely to cause systemic infections (27).

As for the recommended antimicrobial agents for severe *Salmonella* infections, ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol were used for many years. Rising rates of resistance has significantly reduced the efficacy of these agents (114), making fluoroquinolones (e.g., ciprofloxacin) the drug of choice for clinicians in early empirical treatment of severe gastroenteritis in adults (30, 35, 86).

However, quinolone-resistant *Salmonella* strains have already been reported (61, 114), and the emergence of resistance or reduced susceptibility is particularly challenging, since this resistance is caused by mutations in the target enzymes, DNA gyrase and topoisomerase IV (30, 35, 44). Other mechanisms such as increased activity of efflux pumps (EPs), decreased permeability due to loss of porins and a variety of plasmid-mediated quinolone resistance (PMQR) mechanisms also contribute to resistance and/or decreased susceptibility (35).

2. Mechanisms of Action and Resistance to Antibiotics

Antibiotics have been highly effective in the treatment of bacterial infections; however, excessive use for many years has selected drug-resistant strains, creating a worldwide health issue (65, 71), in both medical and agricultural practices (49, 89). In this highly selective environment bacterium harboring resistance determinants obtain an advantage and will survive (109) the bacteriostatic or bactericidal effects of antibiotics and toxic effects of other drugs (71, 88).

Among the major factors determining the frequency of resistance in a bacterial population are (i) the volume of antibiotic use, (ii) the costs of resistance to bacterial fitness, and (iii) the ability of bacteria to genetically compensate for such costs (14).

As for the resistance mechanisms, these can be narrowed down to three main mechanisms. The first one involves the production of enzymes that inactivate antibiotics (42, 71, 89, 108) by hydrolysis or lead to the formation of inactive derivatives (71).

A second mechanism consists in antimicrobial target alteration (42, 71, 89, 108) due to a mutation or enzymatic modification in such a way that the affinity of the antibiotic for the target is reduced (71). Resistance to macrolides, vancomycin, β -lactams, fluoroquinolones and aminoglycosides has been achieved by target-site alteration, while antibiotic inactivation mechanisms have accounted for resistance towards β -lactams, aminoglycosides and chloramphenicol (99). Nevertheless, these mechanisms provide resistance, but only to a single class of compound (99).

The third mechanism of resistance prevents access of the unaltered antimicrobial from reaching its intended target (71, 108, 109) by either a decrease of permeability due to the outer membrane (108) or to the expression of porins (71, 108, 109), or by constitutive/inducible expression of active efflux systems that recognize the antimicrobial and extrude it (11, 89, 108). Porins consist of three outer membrane proteins (OMP) each one of which forms a barrel type structure within the internal cavity, allowing a large variety of hydrophilic compounds to pass through and reach the periplasm. The cell can respond to the presence of noxious agents by down-regulating their number (55). As a second line of defense to agents that manage to reach the periplasm or the cytoplasm, EPs which recognize the noxious agent can rapidly evade

the effects (31, 55) by extruding them to the medium in which the bacterium resides (55).

Multidrug resistance typically results from the accumulation of multiple mutations and/or resistance genes (e.g., on integrons), but specific growth states (e.g., biofilms) and single mutations (e.g., impacting on outer-membrane permeability or the expression of broadly specific multidrug efflux systems) can also promote multi-resistance (89). However, it is the balance between the LPS layer of the outer membrane, general porins and the activity of the main efflux pump (EP) of the organism that generate the intrinsic resistance (2, 54, 109).

2.1. Efflux Pumps

Multidrug resistant (MDR) EPs are cytoplasmic transmembrane proteins (26, 39, 71, 109) found both in Gram-positive and Gram-negative bacteria (39) capable of recognizing and expelling compounds (26, 39, 71, 109), by utilizing cellular energy (71, 109).

Although a variety of mechanisms account for distinct forms of resistance (44, 51, 65, 66), the mechanism now broadly recognized as playing a major role in drug resistance is the over-expression of efflux transporters (47, 51, 65, 97) that (i) extrude a specific substrate or (ii) may transport a range of structurally dissimilar compounds before they reach their target (51, 79, 81, 84, 109).

Antibiotic-specific EPs are usually encoded on transmissible plasmids and transposons (44), while genes encoding MDR pumps are normal constituents of bacterial chromosomes (44, 99). Thus, bacteria have the potential to develop multidrug resistance without acquirement of multiple specific resistance determinants. Genes encoding some MDR pumps are expressed constitutively in wild-type cells (44) and can be considered a “general” resistance mechanism that cooperates with the target mutations, reduced membrane permeability or drug modifications (78). Thus, cells have basal levels of efflux activity, contributing to the decline in antibiotic susceptibility, even though the bacteria might remain susceptible to therapy. However, they would be even more

susceptible if EPs were rendered non-functional, allowing lower doses of antibiotics to be used in therapy (44).

These membrane-spanning proteins involved in the outward transport of a surprisingly large variety of drugs have been recognized and characterized over the last years in almost all cell types, from prokaryotes and archaeobacteria through fungi and higher eukaryotes. They can be classified on the basis of three main criteria, namely the energy source, the phylogenic relationship, and the substrate specificity (10).

2.1.1. Classification of Multidrug Efflux Pumps

Based on bioenergetic criteria, many of these integral membrane proteins can be broadly categorized into two main groups (45, 112), primary and secondary transporters (112). Primary transporters known as ATP-dependent transporters (45) are energized by the hydrolysis of ATP (25, 78, 112). Secondary transporters, on the other hand, harness energy stored in an electrochemical gradient generated by protons that are transported and distributed to the surface of the cell (2, 25, 42, 112), also known as proton motive force (PMF) (45). Membrane proteins coupled to electrochemical gradients provide pathways across the membrane for ion translocation, which occurs via a series of reversible binding events to specific sites (25).

Based on genetic criteria, EP systems of bacteria are classified into five groups (2, 87) (shown in Figure 1) and, as previously described, sub-classified as to the immediate source of protons responsible for energizing the pump (2).

EPs energized by protons under the control of the PMF are classified into four families: the major facilitator superfamily (MFS) (2, 42); the multi-antibiotic toxin extrusion (MATE) family (59, 71); the small multi-drug resistance (SMR) family (50, 62); and the resistance nodulation division (RND) family (75, 111). These transporters differ in size, ranging from about 1000 amino acids for the RND transporters, through 400–450 amino acids for the MFS and MATE transporters and down to 100 amino acids for the SMR transporters (59).

The fifth family – ATP binding cassette (ABC) family – uses the hydrolysis of ATP by ATPase to provide energy for active transport (50, 71, 109).

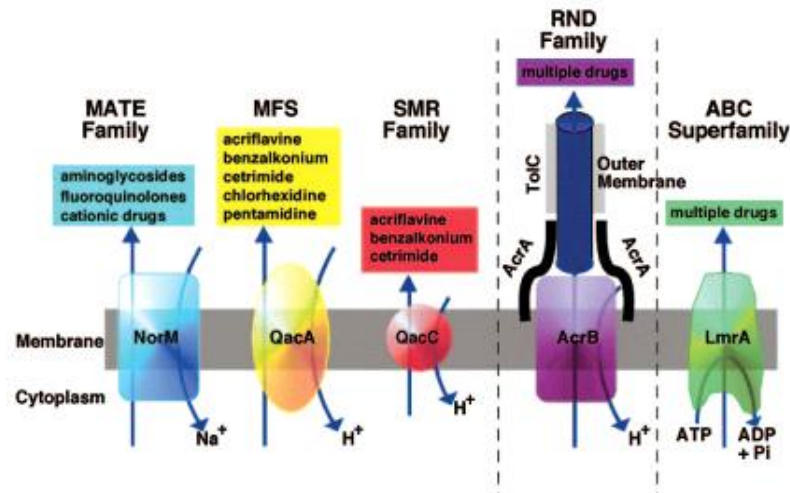


Figure 1 - Diagrammatic comparison of the five families of bacterial EPs (84). Abbreviations: MATE: multidrug and toxic compound extrusion, MFS: major facilitator superfamily, SMR: small multidrug resistance, RND: resistance-nodulation-division, ABC: ATP-binding cassettes.

2.1.1.1. “ATP-binding cassette” (ABC) superfamily

ABC transporters represent ubiquitous membrane systems (50) and constitute one of the largest super-families of proteins known (22) that utilize energy released from ATP hydrolysis (22, 70).

Typically, ABC transporters involved in efflux of toxins, metabolites and drugs (50) appear to be made of four protein domains: two hydrophobic membrane-spanning domains presumed to constitute the translocation channel across the membrane; and two hydrophilic nucleotide-binding domains that interact at the cytoplasmic surface to supply energy for active transport by binding to ATP (22, 50).

These transporters involved in drug resistance are mainly drug specific transporters since a number of them are found in antibiotic-producing organisms, mainly in *Streptomyces* spp., thus ensuring self-resistance to the drugs they produce (50). Specific transporters are also found in *Staphylococcus* and *Enterococcus*, conferring resistance to macrolides and bacitracin (50). In the Gram-negative bacterium *Escherichia coli*, there are approximately 80 ABC transporters (22).

2.1.1.2. “Major Facilitator” superfamily (MFS)

This family is known to represent the largest group of secondary active transporters with well characterized multidrug pumps, including Bmr and Blt of *Bacillus subtilis*, MdfA of *E. coli*, LmrP of *Lactobacillus lactis*, NorA and QacA of *S. aureus*.

The MFS consists of membrane transport proteins that are thought to function as monomers (42). They are found from bacteria to higher eukaryotes and are involved in the symport, antiport, or uniport of various substrates, such as sugars, Krebs cycle intermediates, phosphate esters, oligosaccharides, and antibiotics (50, 92).

These proteins form two separate clusters, with either 12- or 14-transmembrane segments (TMS) (50, 62, 70, 92). MFS pumps with 14-TMS, represented by QacA and QacB, actively extrude monocationic biocides (10, 68) and dyes, such as benzalkonium chloride, cetyltrimethylammonium bromide, and ethidium bromide (68). MFS pumps with 12-TMS, although they have little relevance in the clinic, LmrP of *Lactococcus lactis* pumps out cationic dyes, daunomycin, tetracyclines, and macrolides, and MdfA of *E. coli* can confer increased resistance to cationic dyes, chloramphenicol, and fluoroquinolones when over-expressed from plasmids in a mutant strain lacking the constitutive RND pump AcrB (68).

2.1.1.3. “Multidrug and Toxic Compound Extrusion” (MATE) family

This family is represented by NorM protein of *Vibrio parahaemolyticus* (34, 62), a protein with 12-TMS (70), that actively extrudes various compounds in exchange for the influx of Na⁺ (42, 68), since they act mainly as Na⁺/drug antiporters (34, 50, 70). NorM and its *E. coli* homolog YdhE mediate resistance to dyes, hydrophilic fluoroquinolones, and aminoglycosides (92).

Although there are only about 20 MATE transporters characterized to date, the bacterial genome sequences contain many more examples, and intriguingly, the MATE proteins are present in all kingdoms of life (42).

2.1.1.4. “Small Multidrug Resistance” (SMR) family

Multidrug transporters of the SMR family, the smallest secondary drug efflux proteins known, are typically about 107 amino acid residues in length (92) and contain only four TMS (50, 62, 68, 70). This family contains over 250 annotated members, and is now grouped into three subclasses: the small multidrug pumps, the paired SMR proteins, and suppressors of *groEL* mutant proteins (42).

Due to the small size, it has been proposed that these pumps may function as homo-oligomeric complexes (92), capable of extruding cationic compounds (10, 50, 68) such as quaternary ammonium biocides or ethidium (68). There is only one charged residue in the TMS, Glu14, which has an unusually high pKa of about 8.5. When the transporter encounters the substrate, this residue becomes deprotonated and instead binds the substrate. Thus, the inward flux of proton(s) and the outward flux of the substrate appear to be coupled by the sharing of a common binding site (68).

2.1.1.5. “Resistance Nodulation Division” (RND) superfamily

Drug exporters belonging to RND family, in particular AcrB and its homologues (65) are ubiquitous efflux systems among Gram-negative bacteria (13, 50) that function synergistically with the outer membrane to inhibit the accumulation of toxic molecules in the cell (13).

These efflux systems are capable of recognizing and extruding a wide variety of unrelated compounds, such as antibiotics (42, 51, 65, 108), anti-cancer chemotherapeutics, toxic compounds (e.g.: anionic, cationic, zwitterionic and neutral compounds) (65), biocides, dyes (86) and other noxious agents like bile salts, resulting in a MDR phenotype when over-expressed (47, 51, 68, 108).

This “intrinsic resistance” to lipophilic antibiotics was often thought to be caused by the exclusion of drugs by the outer membrane barrier. However, the inactivation of the major RND pump AcrB of *E. coli* makes the bacteria almost completely susceptible to these agents even in the presence of the intact outer membrane barrier (69). Thus, the characteristic intrinsic resistance of Gram-negative bacteria to these antibiotics owes as much to the RND pumps as to the outer membrane barrier (68).

As shown in Figure 2, these transport systems form tripartite protein complexes (28, 42, 52, 84) rather than a single transport protein (59). The RND pump in *E. coli* and other Gram-negative bacteria are composed of three components: AcrB – an inner-membrane protein (IMP) transporter (52, 59, 68, 79, 84) that captures substrates within either the phospholipid bilayer of the inner membrane or the cytoplasm (84, 106), TolC – an outer membrane protein (OMP) channel that traverses the cell envelope providing a conduit to the exterior, and AcrA – periplasmic membrane fusion protein (MFP) that anchors TolC to the plasma membrane (52, 59, 68, 79, 84), allowing the cooperation between AcrB and TolC (65, 84). In *E. coli*, TolC has the ability to interact with different classes of inner-membrane proton antiporters, which may belong either to the RND family or the MFS (26).

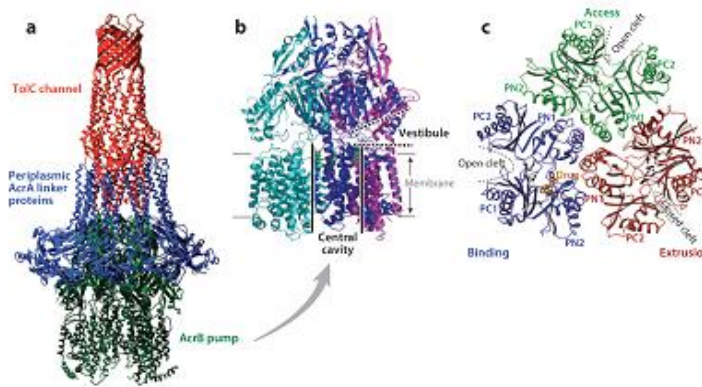


Figure 2 - Schematic representation of the tripartite efflux system AcrAB-TolC (a) The model of the AcrB-AcrA-TolC tripartite complex that exports drugs directly into the medium. (b) AcrB trimer. Each protomer is shown in a different color. The large central cavity is connected to the periplasm through vestibules between protomers. The proximal portion of the structure was cut away to reveal the presence of vestibule. (c) The periplasmic domain of the asymmetric AcrB trimer viewed from the top. The conformation of each protomer is characteristic, with open or closed external clefts. A drug molecule is seen bound to the binding protomer (68).

The AcrAB-TolC system is highly homologous to the MexAB-OprM RND system in *P. aeruginosa* (70, 84). AcrA is a 397-amino acid protein that interacts with AcrB, a much larger protein with 1,048 amino acids (84). TolC, a 506-amino acid (84) homotrimeric exit duct, that looks like a uniform tube with a 40Å thick β -barrel penetrating the outer membrane (66, 78) and a 100Å long periplasmically protruding tube surrounding six coiled coils (66), which interacts with the periplasmic adaptor and/or the inner-membrane transporter leading to the conformational changes (78, 84). AcrA is

presumed to be a highly elongated molecule that can span the periplasmic space, suggesting that AcrB might directly dock with TolC and that AcrA might reinforce the docking at the side of the complex (66). These transporter proteins are composed of 12-TMS with two large periplasmic domains that possibly provide substrate specificity (Figure 3) (50).

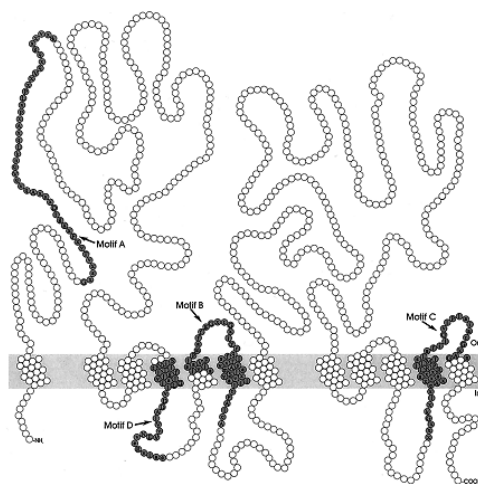


Figure 3 – Structural model for multidrug transporters of the RND family. The residues constituting the conserved sequence motifs are shaded (92).

This construction allows the direct and efficient export of drugs into the external medium, rather than into the periplasmic space (10, 50, 68, 70). This provides a huge advantage for bacterial cells since once exported into the external space drug molecules must traverse the outer membrane barrier to reenter bacterial cells. Thus, these pumps work synergistically with the outer membrane barrier (68, 69) that acts as an additional protective step limiting the passive penetration of hydrophilic and hydrophobic molecules (47).

Also, substrates might access the AcrB central cavity either from the cytoplasm through the trans-membrane region or from the periplasm through the AcrB lateral vestibules (66, 68, 78, 81). This was predicted because carbenicillin, which cannot cross the cytoplasmic membrane owing to the presence of two carboxylate groups, is still a good substrate for these pumps (68, 81). This allows the pumps not only to prevent the entry of drugs into the cytoplasm, but also to extrude agents, such as β -lactams, whose targets are on the outside surface of the cytoplasmic membrane (66, 68, 81). Importantly, the

periplasmic capture mode allows the RND tripartite pumps to collaborate synergistically with simple EPs that extrude substrate drugs only into the periplasm (68).

As previously described, RND pumps are proton antiporters (52, 65, 84), that utilize the trans-membrane proton gradient (26) across the membrane to power efflux, exchanging one H^+ ion for one drug molecule (52, 84). The membrane surface is separated from the bulk aqueous phase by an electrostatic barrier of ions (63). This gradient provides the protons present in the periplasmic space and, when they enter the transporter at its plasma membrane base, they energize the pump (2, 52) and the agent which is believed to be concentrated within the inner membrane is in turn extruded. The proton is then released to the medial side of the plasma membrane. The fusion proteins are believed to physically assist the extrusion of the agent (52).

The PMF is maintained by the metabolic activity of the bacterium and is in part established by protons generated in the inner membrane following the hydrolysis of ATP (54) catalyzed by membrane bound ATPases and by oxidative metabolism (52).

After the protons are exported to the periplasm by a variety of transport processes (2, 52), most are then exported to the surface of the cell, distributed on its surface and bound to reactive groups of the outer cell wall (LPS and $-CO_2^-$) (54), creating a proton gradient that is greatest at the surface of the cell and least in its periplasm (2, 52). The proton translocation is driven by membrane-associated proteins that couple electron flow from low-redox potential electron donors to higher-potential electron acceptors (95). This distribution establishes a relative negative charged periplasmic space and positive charged surface of the cell, resulting in an electrochemical gradient (2, 52, 54), which can be maintained at extreme pH and temperature (2).

The resulting trans-membrane difference in the electrochemical potential of hydrogen ions was at first seen to be the driving force behind the energy consuming enzymes and ATP-synthase and was defined as the proton motive force (PMF). However, because of the largesse of the bulk water phase the dissemination of protons from the surface of the cell would quickly eliminate the pH gradient across the cell envelope, and hence, the PMF and the energy it provides for driving EPs would be eliminated. Because the PMF

is maintained by the bacterium when challenged by changes in the pH of the environment, the chemiosmotic theory should possibly include the concept that the distribution of protons on the surface of the cell which due to the lipopolysaccharide components of the outer cell envelope, would be selectively concentrated (2, 52) and result in a pH of the medium immediately surrounding the surface of the cell that would be much lower than the pH of the bulk medium (52, 54). This surface distribution of protons therefore assists the bacterial cell in maintaining a PMF that would not be significantly affected by the pH of the medium (52). In conclusion, the PMF is therefore composed of a chemical proton gradient (ΔpH , inside alkaline) and an electrical potential ($\Delta\Psi$, inside negative) (92).

2.1.2. Efflux systems in *E. coli* and *Salmonella enterica*

These transporter proteins thought to play a crucial role in antibiotic resistant food-borne pathogens are isolated both from humans and from animals capable of spreading from one ecosystem to another (84).

Drug efflux systems in *E. coli* have been used as models for genetic and biochemical studies (42). The complete genome sequence of *E. coli* was determined by Blattner et al. in 1997 and its analysis revealed 37 putative drug transporter genes from the five families: 19 of the MFS, 3 of the SMR, 7 of the RND, 7 of the ABC, and 1 of the MATE (70). The transport systems of these genes were classified according to the putative membrane topology, protein family, bioenergetics, and substrate specificity (82).

In *E. coli*, there are approximately seven different proton-dependent MDR pump systems that can export structurally unrelated antibiotics (101, 106): AcrAB-TolC, EmrAB, MdfA, TehA, EmrE, AcrEF, and EmrD (101). All of the above fall into three distinct families, encoded by the following genes: the major facilitator superfamily (MFS: *emrD*, *mdfA*, *emrB*), the resistance nodulation-cell division family (RND: *acrB*, *acrF*, *acrD*, *yhiV*), and the small multidrug resistance family (SMR: *emrE*, *tehA*) (101, 106). Putative ATP-dependent drug EPs in *E. coli* have also been identified by Paulsen et al. (82), including YhiG, MdlB, YbjZ, and MsbA (101).

Even in the presence of a variety of MDR pumps, with respect to *E. coli*, extrusion of noxious agents is performed primarily by the over-expression of the *acrAB* locus encoding the multidrug EP AcrB, controlled by the *marRAB* operon, although other EPs may assist in the extrusion process (52, 85).

As mentioned earlier, the *E. coli* AcrB can pump out a very wide range of compounds, including lipophilic drugs, detergents (including bile salts), dyes and even solvents (as seen in Table 1) (68, 84, 97).

Table I – Bacterial EPs involved in antibiotic resistance by *E. coli* and respective characteristics. Properties EPs are shown together with the periplasmic adaptor protein (MFP) and the outer membrane channel (OMF) they interact with (68, 69, 84, 89).

RND pump	Associates with:		Substrates	Regulatory Genes
	MFP	OMF		
AcrB	AcrA	TolC	AC, AH, BA, BL, BS, CM, CV, EtBr, ER, FA, FQ, FU, LN, ML, MX, NV, organic solvents, penicillins, PO, SDS, TC, TP, TS, TX	<i>acrR, marA, robA, soxS, sdiA</i>
AcrD	AcrA	TolC	AG, DOC, FU, NV, SDS, BL	<i>sdiA, baeR, cpxR, ompR</i>
AcrF	AcrE	TolC	AH, FQ, TC, TP, LN, ML, BS, SDS, AC, RD, BL	<i>acrS, sdiA</i>
MdtB / MdtC (YeN/ YegO)	MdtA (YegM)	TolC	NV, BL, BS, SDS	<i>baeSR</i>
YhiV	YhiU	TolC	ER, NV, DOX, CV, EtBr, SDS, BS, BA, RD, BL	<i>evgA</i>

Legend: AC, acriflavine; AG, aminoglycosides; AH, aromatic hydrocarbons; BA, benzalkonium; BL, b-lactams; BS, bile salts; CH, cholate; CM, chloramphenicol; CV, crystal violet; DOC, deoxycholate; DOX, doxyrubicin; ER, erythromycin; EtBr, ethidium bromide; FA, fatty acids; FQ, fluoroquinolones; FU, fusaric acid; LN, linezolid; ML, macrolides; MX, methotrexate; NAL, nalidixic acid; NOR, norfloxacin; NV, novobiocin; PO, pine oil; RD, rhodamine; RF, rifampicin; SDS, sodium dodecyl sulphate; TC, tetracycline; TP, trimethoprim; TS, triclosan; TX, Triton X-100.

E. coli over-expressing AcrAB demonstrates increased resistance to substrates such as bile salts, when compared to an isogenic wild-type strain; and similarly, a strain with an *acrAB* deletion is more susceptible than the wild-type strain to the same compounds (101).

The expression of homologs such as AcrD can become increased through regulatory responses or mutations (68) in the presence of aminoglycosides which they are capable of extruding (a function that could not be carried out by the AcrB pump) (68, 84).

The AcrE and AcrF pumps also mentioned above are 80 and 88% similar to AcrA and AcrB, respectively (84). In particular, the AcrEF-TolC system can extrude similar substrates (e.g., lipophilic and amphiphilic agents (97)) albeit at lower efficiency levels. Moreover, these pumps can be over-expressed when AcrAB is deleted or inactivated (79). Therefore, *acrAB* or *acrEF* deletion mutants are increasingly susceptible to a wide variety of antibiotics and detergents and could be made significantly more resistant to these substances by the respective insertion of *acrAB*- or *acrEF*-carrying plasmids (106). However, although increased expression of AcrEF results in increased resistance to some AcrAB substrates, deletion of *acrEF* does not contribute to increased susceptibility to those substrates (101), demonstrating that the AcrAB-TolC pump is the dominant MDR pump among other pumps (65, 101).

Even so, it should be again noted that the over-expression alone of the AcrAB-TolC system is unlikely to give rise to clinically significant levels of resistance (84). For example, fluoroquinolone resistance in *E. coli* can be caused by mutations in the target topoisomerases of the drugs, DNA gyrase (e.g., in *gyrA*) and topoisomerase IV (e.g., in *parC*), or they can affect regulatory genes such as *marA* or *soxS*. Overexpression of *marA* causes decreased expression of the OmpF porin as well as increased expression of the multidrug EP AcrAB, thereby conferring resistance (42, 73). A mutation in a topoisomerase gene which affects the capability of fluoroquinolones is unlikely to give rise to clinically significant levels of resistance; however, when combined with an over-expressed AcrAB-TolC system, such isolates are resistant to the breakpoint concentration of ciprofloxacin (84).

Efflux may cooperate with other resistance mechanisms to confer not only high level but also broad-spectrum resistance. In *E. coli*, expression of class-C β -lactamase confers resistance to first- and second- generation cephalosporins, and expression of pump AcrB causes resistance to most penicillins. The global result is that the organism becomes susceptible only to third- or fourth- generation cephalosporins (11).

On the other hand, *Salmonellae* also possess multiple drug efflux systems (42). At least nine drug EP genes exist in *Salmonella enterica*, eight of which are also found in *E. coli* (71).

Due to efflux mechanisms, adaptive resistance to a variety of compounds like benzalkonium chloride and triclosan, and to agents used in treating infections in poultry like fluoroquinolones, β -lactams, macrolides (e.g., erythromycin), and tetracycline are substrates for MDR EPs identified in *Salmonella* spp (42, 84).

Previous studies have discovered that *Salmonella* responds to acidic challenges through an adaptive system called the acid tolerance response in which adaptation to mild acid conditions enables the organism to survive periods of severe acid stress (83). Ingested *Salmonella* must survive the acidic environment of the duodenum, which receives bile salts that are toxic to the organism. When it reaches the colon, the pH is near neutral but due to the presence of bile salts the environment remains toxic (5).

The means by which the organism escapes injury from bile salts is by the activity of its AcrAB–TolC EP, which extrudes the noxious compounds prior to their reaching their intended targets. It has been suggested that as long as the organism is located in the colon, the activity of the AcrAB–TolC EP also protects it from antibiotics, rendering therapy of salmonellosis a serious problem (5).

Additional studies demonstrate that when either the *acrD* or *acrF* genes are deleted, the AcrB expression is increased; likewise, when *acrB* gene is deleted, expression of AcrD or AcrF increases. However, a double-knockout mutant lacking AcrB and AcrF is no more susceptible than a construct lacking AcrB alone. These data suggests that the major EP in *S. enterica* serovar Typhimurium, and probably all serovars of *S. enterica*, is again the AcrAB-TolC pump (5, 84).

The ability of *Salmonella* to cause infection in humans is also due in part to its ability to survive within the phagolysosome of the neutrophil (21). Survival within the phagolysosomal compartment results from activation of the two-component regulatory system PmrA/B (21, 83), an operon that involves at least nine genes resulting in the synthesis and insertion of lipid A into the nascent lipopolysaccharide layer of the cell envelope. This response renders the organism resistant to enzymatic digestion, to other antimicrobial proteins as well as many antibiotics (21).

2.1.3. Efflux pump systems: Physiological Importance

Despite compelling evidence implicating EPs in multidrug resistance, the ongoing debate concerning the “natural” function of these proteins has not abated (71, 89). Clearly the RND-type multidrug pumps have not appeared in response to the widespread use of antibiotics and for drug resistance (42, 68), as most of these pumps are coded by chromosomal genes that are present in strains isolated long before the antibiotic era (68).

As discussed previously, MDR EPs can recognize a wide range of structurally unrelated substrates including those compounds produced by higher organisms, such as bile salts (42, 71), fatty acids and hormones (42), and extrude them prior to its reaching sensitive and lethal targets within the bacterium (52, 68). They can also respond to a range of stimuli including stress signals, and influence the colonization (42), pathogenesis or virulence (42, 71), cell communications, biofilm formation and other fitness responses. These functions may well be the physiological functions of at least some of the drug pumps, and may ensure the persistence of drug efflux transporters in evolution. Thus, pumps are likely to affect the interaction of bacteria with its host (42).

The observed inducibility of the *E. coli* AcrAB system by toxic fatty acids, and the demonstrated role of AcrAB in the export of and resistance to bile salts (86, 89) (present the highest affinity, among substrates, to the AcrB transporter (68)), have been cited as support for a role in protecting the cell from the action of these agents in the gut (86, 89).

Also, exposure of *Salmonella* to low pH is well known to activate the PmrA/PmrB, a regulatory system which not only allows the organism to survive the low pH of the phagolysosome, but also increases its resistance to antibiotics. Exposure of *E. coli* to low pH activates a wide spectrum of genes, some of which code for cell envelope proteins. These studies suggest that low pH readily activates genes of a Gram-negative bacterium and render the organism resistant (52).

Export of bacterial products is also a proposed function for these multidrug efflux systems, with a previous review implicating byproducts of metabolism as probable substrates for these efflux systems. The up-regulation of the *E. coli* AcrAB efflux system in strains with mutations in central biosynthetic pathways, possibly as a result of accumulation of pathway intermediates, certainly supports this idea (89).

Understanding the physiological roles of the MDR pumps may continue to be rather difficult as the functions of these pumps are often involved in a complex, and overlapping network of reactions in the bacterial cell (42). There is clearly a lack of evidence for the induction of efflux gene expression by antibiotics (86, 89), despite the fact that most of the pumps are regulated, often by the product of a linked regulatory gene (87). If antibiotics are not the 'signal' for pump expression, elucidating this signal is likely to be very useful in discerning the natural function of these efflux mechanisms (87).

2.1.3.1. Importance of Calcium Regulation

Over the years, a number of bacterial proteins have been identified to be involved in calcium transport mechanisms. Like eukaryotes, bacterial cells have ion channels (Ca^{2+} , K^+ , Na^+), primary and secondary transporters, and Ca^{2+} -binding proteins (CaBPs), which may be involved in Ca^{2+} homeostasis (23, 110). The idea that Ca^{2+} acts as a messenger is based on the concept that environmental signals induce changes in the intracellular free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (23), in order to control many cellular processes in eukaryotic transport systems (37).

Much evidence supports the role of Ca^{2+} as a regulator in prokaryotes however, the extent and importance still remains unclear. It is known that bacteria keep tight control of their $[\text{Ca}^{2+}]_i$ with values very similar to those found in eukaryotes (100–300 nM) (23, 105).

There is data that supports the involvement of calcium in a number of bacterial processes such as maintenance of cell structure, motility, transport, cell division, gene expression and cell differentiation processes such as sporulation, heterocyst formation and fruiting body development (23). It is vital for signaling and activating genetic systems as well as a broad range of metabolic and energy providing pathways within the cell (67, 72). Additionally, the growing number of proteins containing various Ca^{2+} -binding motifs supports the importance of Ca^{2+} , which controls various protein functions by affecting protein stability, enzymatic activity or signal transduction (60).

Recently, Jones et al showed that the periplasm can accumulate Ca^{2+} ions three- to sixfold when compared to the external medium by performing $[\text{Ca}^{2+}]_i$ measurements in the *E. coli* periplasm (33). These findings suggest that both the outer membrane and the periplasm could be crucial for Ca^{2+} regulation by acting as Ca^{2+} barriers or buffering and sorting Ca^{2+} , thereby regulating the availability of free- Ca^{2+} to Ca^{2+} transporters in the inner membrane (23, 33).

3. MDR Efflux Inhibitors

The expanding problem of MDR EPs and their ability to decrease intracellular concentration of active antibiotics has required the development of new therapeutic treatments (13). In order to address this problem, it is necessary to search for and develop new molecules to circumvent efflux activity (47), either by modifying existing antibiotics or by discovering and developing therapeutic agents which inhibit the transport activity of EPs and could be used in combination with existing antibiotics (44).

To specifically block the activity of drug EPs, there are different possibilities (i) avoid efflux activity and thus, enhance the activity of multiple substrates by reducing their

efflux; (ii) decrease the permeability efficacy of the membrane barrier (44, 47); (iii) block the efflux capacity of bacterial cell by altering the pump function (47, 78).

Regarding the approach based on the inhibition of EPs, the antibiotic is co-administered with an inhibitor that neutralizes efflux based resistance, rendering the antibiotic useful, even in resistant organisms (50, 13). Such molecules, the efflux pump inhibitors (EPIs), are expected to: (i) decrease the intrinsic resistance of bacteria to antibiotics by increasing the intracellular concentration, (ii) reverse acquired resistance even in highly resistant strains with multiple target mutations, (iii) and reduce the frequency of emergence of resistant mutant strains (50, 47). The net result of blocking the efflux of an antimicrobial compound by the use of EPIs is to decrease the threshold concentration (i.e. the minimum inhibitory concentration (MIC)) of the antimicrobial compound (13).

Several classes of compounds that block efflux by the RND efflux systems have been identified in screens of chemical libraries (50, 13) or in biodiversity-issued compounds, which could be further optimized by structure–activity relationships studies (50) and therefore potentiate antibiotics that are RND efflux substrates (13).

Nevertheless, while very compelling scientifically and of clear potential clinical benefit, this approach brings with it many challenges. Besides the obvious issues common to all infectious disease drug development programs (appropriate potency, spectrum of activity, bioavailability, clearance, toxicity, etc.), several are specific to this strategy. In the discovery phase, the pharmacokinetics of the EPIs must be taken into account and viable pharmacokinetic profiles of the potentiator may be significantly restricted as a result. In development, clinical trials must be designed to demonstrate improvement of a combination product over existing therapies (44).

The search for newer antimicrobials has also prompted studies demonstrating that many medicinal compounds belonging to various pharmacological groups, such as tranquilizers, antihypertensives, antipsychotics, non-steroidal anti-inflammatory agents (NSAIDs), mucolytic agents, calcium channel blockers, cardiovascular drugs, proton pump inhibitors and even antispasmodic agents have some influence on the physiology and on the viability of microorganisms, thus the name ‘non-antibiotics’ (21, 64). The

use of these drugs in combination with antibiotics possess the advantage of being cost-effective and time-saving, since a lot of data is already available, with respect to toxicity and pharmacokinetics (50).

Some EPIs have been identified over the past decades, including phenylalanyl arginyl- β -naphthylamide (PA β N) verapamil, phenothiazines (e.g., thioridazine (TZ) and chlorpromazine (CPZ)), and compounds isolated from plants (55). However, to date no EPI has been licensed for use in the treatment of bacterial infections in human or veterinary medicine, and it is clear that this gap must stimulate research that leads to the development of new EPI molecules (47).

3.1. Calcium channel inhibitors

3.1.1. Phenothiazines

Phenothiazines, a group of antipsychotic drugs commonly used to treat certain mental disorders (37) due to the neuroleptic properties of methylene blue (4, 104), were found to interact strongly with calmodulin, a Ca^{2+} -binding protein that modulates a number of proteins regulating cellular functions (37). These calmodulin antagonists and other related compounds inhibit the transport of Ca^{2+} and K^{+} since they inhibit the binding of Ca^{2+} to calmodulin or calmodulin-type proteins (4, 55). The inhibition of calcium access to Ca^{2+} -dependent ATPase inhibits transport processes such as those performed by influx and EPs (55). Since phenothiazines specifically inhibit binding of calcium to calcium-dependent enzymes, they inhibit their activity and, hence, the transport processes (54, 55).

The potential use of phenothiazines as antibacterial agents or as enhancers of antibiotic activity lies in their ability to kill phagocytosed bacteria, namely in *Mycobacterium tuberculosis* and *Staphylococcus aureus*. This occurs due to the ability of the macrophage to concentrate the phenothiazine to a level comparable to a minimal bactericidal concentration (4). Thus, Gram-positive cocci, Mycobacteria and some Gram-negative rods, such as *Shigella* spp., are more susceptible to the antibacterial properties of a number of phenothiazines, opposed to Gram-negative rods such as *Escherichia coli* and *Salmonella* spp. (4).

However, phenothiazines can interfere by other means. In Gram-negative bacterium such as *E. coli*, β -lactams specifically bind to penicillin-binding protein (PBP) 3, which is associated with the filamentation of the bacterium. Since phenothiazines also affect filamentation, they may either bind directly to a PBP or have some effect on other mechanisms that indirectly influence the PBP.

It is worth noting that filamentation of Gram-negative bacteria can also be produced by non- β -lactam antibiotics (e.g., quinolones), as well as by physical conditions (e.g., release from hydrostatic pressure) and by growth conditions, therefore filamentation caused by sub-inhibitory concentrations of a phenothiazine may not involve a direct effect on the PBP itself (4).

With respect to the adherence of Gram-negative bacteria, phenothiazine Promethazine has shown the ability to reduce the effect on epithelial cells. It prevents the recurrence of pyelonephritis caused by *E. coli* and, since the concentration required to inhibit the growth of bacteria is well beyond that clinically relevant, the successful therapy of recurrent pyelonephritis is possibly due to the effect it has on the adherence of *E. coli* to the epithelium of the urinary bladder (4). It seems plausible that phenothiazines inhibit adherence by inhibiting pili formation, which is also true with low concentrations of antibiotics, as well as by interfering with access of bacterial pili to receptors present on the surface of the epithelial cell (4).

3.1.1.1. Chlorpromazine

Chlorpromazine (CPZ) was introduced by Rhone-Poulenc in the early 1950s (4, 64, 76) as a neuroleptic drug employed for the management of psychosis (3). CPZ as well as other phenothiazines such as thioridazine (TZ), promazine, promethazine and desipramine present antimicrobial properties *in vitro* against a variety of bacteria (4, 76) due to its capacity to affect the ultra-structure of the cell wall (3).

CPZ inhibits the binding of calcium to calcium binding proteins of eukaryotes and prokaryotes. As a consequence of its inhibitory properties, CPZ also inhibits bacterial kinases and phosphatases, calcium and potassium transport (2).

CPZ is concentrated in excess of 100-fold by tissues and organs rich in macrophages and *in vitro* concentrations that are within clinical reach are effective against phagocytosed organisms (76). Studies have also shown that CPZ has the ability to inhibit the *in vitro* growth of mycobacteria, inhibit the secretion of toxins, cause the elimination of plasmids from infected Gram-negative bacteria, cause the lysis of bacteria, increase the permeability of bacteria to agents, reverse resistance to antibiotics and inhibit bacterial enzymes (2).

When *E. coli* is exposed to sub-MIC levels of CPZ, regardless of its resistance to CPZ, it will exhibit elongation and of significant importance, exhibit far greater sensitivity to antibiotics to which it is sensitive, lowering the MIC values (3). The effects of CPZ on *E. coli* were noted in the protein composition of the plasma membrane; alterations which may be related to the inhibitory effects of CPZ on the EP which in turn was postulated to result in greater retention of antibiotic within the cytoplasm, enhancing antibiotic activity with the co-presence of CPZ (3).

However, because prolonged use of CPZ produced a number of serious side-effects (4), and whatever antimicrobial activity reported was essentially one produced only *in vitro* at concentrations beyond clinical reach (4, 76), CPZ and other phenothiazines were not seriously considered as potential sources of new antibiotics, even when they were shown to have desired antimicrobial effects *in vivo* (4).

3.2. Novel Compounds

Given the development failure of new antibiotics and the emergence and spread of MDR bacteria, there is a vital need to identify clear targets to block efflux mechanism, and select molecules with a high inhibitory efficacy and no toxic effects on mammal cells, a field that remains undefined and vague (115).

While resistance to naturally-occurring antibiotics is prevalent in the soil environment, genes conferring resistance do not seem to take over and fixate in these natural populations; instead resistant and sensitive bacterial strains coexist. Possibly, while antibiotics select for resistant strains, other natural mechanisms may perhaps exist to

choose against resistance (80). This ability to select for or against resistance depends on the selective pressure the new compound applies and on the duration of its activity, determined by its chemical stability (80).

In the natural environment, antibiotics are not static - a single drug can decay into a range of compounds, each accumulating and degrading with different kinetics and displaying different selective effects (80). Due to the short-life of antibiotics, they decay to an assortment of chemical species which may be more stable than the precursor drug, and may therefore have significant ecological impacts. Thus, competition between antibiotic resistant and sensitive strains may be influenced both by the short-term effect of an antibiotic and by the potential long term effects of its degradation products (80).

3.2.1. Hydantoin compounds

Many hydantoins derivatives find important applications as medicinal (anticonvulsant drugs in the treatment of epilepsy, anti-arrhythmic (7, 8, 9), antitumor, anticancer (7, 91), anti-inflammatory (8, 9, 91), anti-muscarinic, anti-ulcers, anti-viral, anti-diabetic (8) and antiandrogens drugs (9)) and as agrochemicals (bactericides, fungicides (7) and as aldose reductase inhibitors (9)).

As an anticonvulsant drug, it is very effective in controlling a variety of seizure disorders while impairing little neurologic function, if at all (20). This modulation of the neurologic function is believed to be due to alteration of membrane conductance of Na^+ and Ca^{2+} ions, whose conductance are important during the function of excitable nerve, heart, and muscle tissues. Early work suggested the hypothesis that the effects were due to a selective block of high-frequency neuronal activity by selective modulation of eukaryotic ion-driven EPs (20). As an antimicrobial, hydantoin compositions are generally added to industrial aqueous fluid media to reduce or inhibit the growth of microorganisms (48).

Hydantoins and thiohydantoins are compounds that possess a reactive urea core. They are an important structural moiety found in several natural products and pharmacologically important compounds (9). In recent years, considerable efforts have been devoted to the development of novel and more efficient methods for synthesis of

(thio)hydantoin derivatives (7), due to their importance as substrates for enzymatic reactions (1).

Synthetically, hydantoins are important precursors of amino acids, via either acid-, base- or enzyme-catalyzed hydrolysis. The Bucherer–Bergs reaction is the most commonly used method for the synthesis of hydantoins. This multi-component reaction starts from an aldehyde or a ketone whose ready availability makes the Bucherer–Bergs reaction an attractive method for the synthesis of hydantoins. However, the use of potassium cyanide (KCN) leads to safety problems, causing the reaction to be conducted within a sealed tube at a temperature of 80°C. Other methods furnishing hydantoins include the treatment of amino amides with triphosgene, the reaction of amino acids with acetic anhydride and ammonium thiocyanate (to create the thiohydantoins), the combination of carbodiimides with unsaturated carboxylic acids, and the treatment of nitriles with organometallic reagents followed by addition of potassium cyanide and ammonium carbonate (9).

Within certain species of bacteria, the hydantoin substrate compounds are first hydrolyzed by hydantoinase enzymes in a ring-opening step (1, 24, 102) to form either L- or D- *N*-carbamoyl- α -amino acids (i.e. hydantoic acids) (1, 24, 93, 102). Hydantoin-cleaving enzymes can often be found in combination with highly stereoselective *N*-carbamoyl- α -amino acid amido hydrolase (*N*-carbamoylases) enzymes, which catalyze the further hydrolysis of the hydantoic acids to the free D- or L-amino acids in an irreversible reaction (1, 102), as presented in Figure 4.

Together, these microbial hydantoin-hydrolysing enzyme systems (32) accomplish the total conversion of D, L-5-monosubstituted hydantoin compounds to produce optically pure D- or L-amino acids (1, 32, 93, 102), important intermediates for the production of various drugs and pharmaceuticals (32, 102). For example, D-Hydantoinase, together with *N*-carbamoyl-D-amino acid hydrolase is used in the production of D-amino acids for the synthesis of semi-synthetic antibiotics, peptide hormones, pyrethroids and pesticides (24).

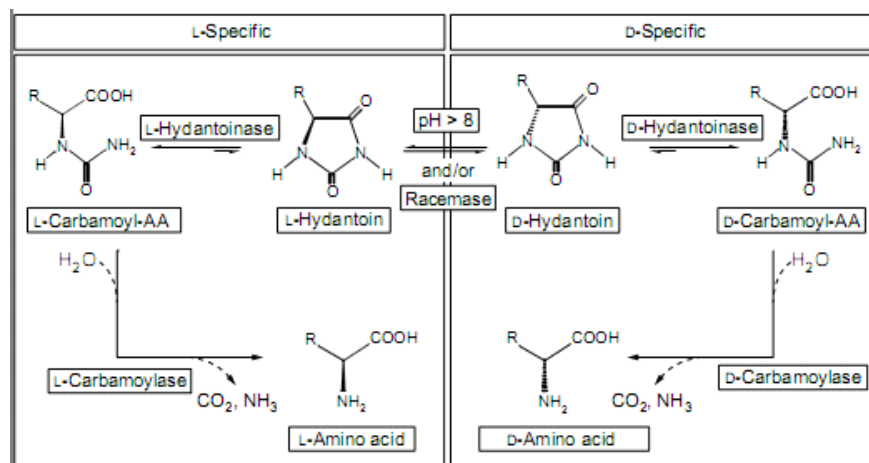


Figure 4 - Reaction scheme for the enzymatic cleavage of D, L-5-monosubstituted hydantoin derivatives to the corresponding D- or L-amino acids. AA: amino acid (1).

The most common anomalies seen in patients exposed to hydantoins occur during pregnancy with deficiency in growth. Cleft lip and palate, developmental delay, mental retardation, and mid-face hypoplasia are also supportive of the diagnosis. Exposure of the embryos to the action of hydantoin decrease the embryo's survival rate and weight and induce neural development retardation and abnormal morphogenesis (20).

Nevertheless these compounds are considered to be good candidates for new EPIs since their mechanism of action is similar to the one described for other known eukaryotic EPIs such as the phenothiazine group, with potential to be used to inhibit efflux activity of prokaryotic EPs (46, 98).

4. Detection methods of accumulation and efflux in bacteria

4.1. Semi-automatic fluorometric method to monitor ethidium bromide transport through bacterial membranes

A semi-automated fluorometric method recently developed by the Group of Micobacteria of the Unit of Microbiology of the IHMT/UNL, uses the universal EP substrate EtBr for the real-time assessment of EP activity in bacteria (2, 31, 96, 108). To enable this, the method employs the Rotor-Gene TM 3000 thermocycler (Corbett

Research) capable of evaluating the EP activity *in vivo* and on-line of a large number of samples, with the possibility to expose the same cell preparation to different physiological conditions in a single assay (2, 79) (e.g., temperature, pH, presence and absence of the energy source) (108).

This technique is sustained by the premise that if the EP is inhibited by potential EPIs undergoing evaluation, antibiotics which are extruded by this system and which have become ineffective may again be useful (109).

EtBr is a substrate widely used by bacterial EPs and susceptibility to it in the presence and absence of an EPI has been used as a screen for the presence of efflux-related resistance mechanisms (31). EtBr emits weak fluorescence in aqueous solution (external to the cell) and becomes strongly fluorescent when concentrated in the periplasm (79, 96) of Gram-negative bacteria and in the cytoplasm of Gram-positive bacteria (96). As long as EtBr is not intercalated between nucleic bases of DNA, it is subject to extrusion serving as a useful probe. When intercalation into DNA results in a strong binding, it precludes EtBr from having access to the EP system of the bacterium (79, 96).

Accumulation of EtBr inside the cell is the result of the interplay between cell-wall permeability and efflux activity. The fluorescence that results from the overall intracellular EtBr content is monitored by real-time fluorometry (96). This method makes it possible to distinguish accumulation, which reflects the balance between influx and efflux, from efflux itself and, therefore, has the potential for the study of the kinetics of EtBr transport (79). The determination of the influx and efflux rates of the fluorescent substrate EtBr allows the quantification of the cell's overall efflux capacity, providing useful information to interpret different phenotypes resulting from this activity, including multidrug resistance in clinical bacterial strains (79).

In Figure 5 we can observe a simplified schematic representation of a graphic obtained by the fluorescence assay in which a bacterial strain is subjected to the presence and absence of a potential EPI. Figures 5B) and 5C) show the influx and efflux of fluorescent substrate EtBr by EP AcrAB-TolC when a bacterial strain is subjected to the presence and absence of a potential EPI.

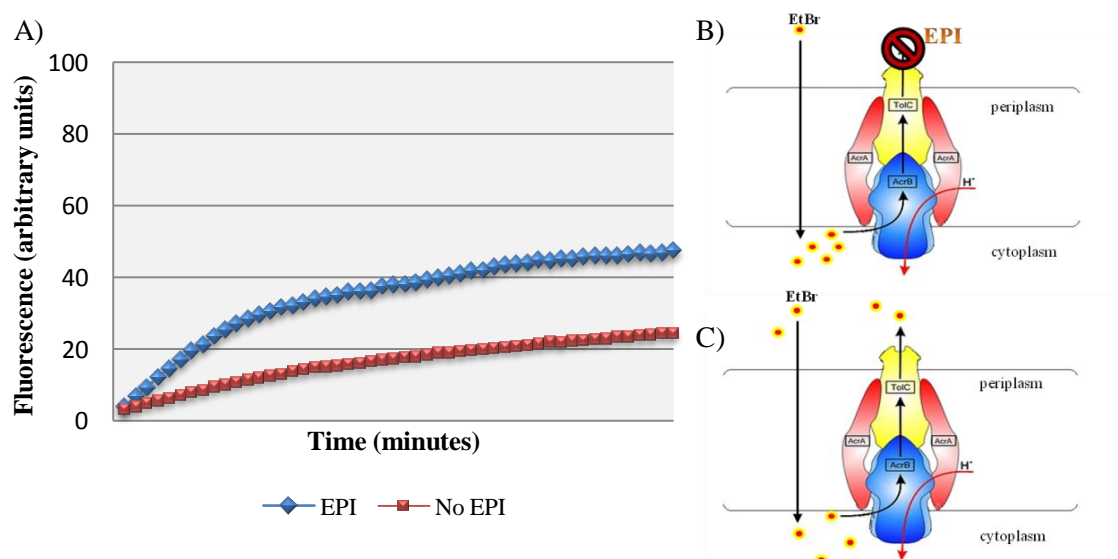


Figure 5 – Accumulation of substrate EtBr in presence and absence of a potential inhibitor of EPs. A) Example of a possible graph obtained by the fluorescence assay in which a bacterial strain is subjected to the presence and absence of a potential EPI; **B)** Schematic representation of the accumulation of EtBr in a bacterial cell due to the presence of an EPI (rate input > output rate); **C)** Schematic representation of input and efflux of EtBr in a bacterial cell in the absence of an EPI (rate input = output rate); **Legend:** EPI (efflux pump inhibitor), EtBr (ethidium bromide), EPs (efflux pumps).

II. STUDY OBJECTIVES

Active EPs in prokaryotic cells strongly modulate the activity of a large number of antibiotics. In order to better understand multidrug resistance and the mechanisms that trigger this condition, in this study we intend to expose *Salmonella* NCTC strain to newly synthesized hydantoin compounds in the presence of universal EP substrate EtBr, and differences in the accumulation/retention of that substrate by the selected strain in the presence of these inhibitory efflux compounds are to be expected. If this takes place, we will proceed in exposing in vitro-derived ciprofloxacin-resistant mutants *Salmonella* 104-CIP and *Salmonella* 5408-CIP, induced to high level resistance by step-wise exposure to this antibiotic and over-expressing EPs, to all the compounds that have favorable results. This way, we can observe if the compounds are indeed effective efflux inhibitors when in the presence of strains with induced resistance by overexpression of efflux pumps.

In addition to the evaluation of newly synthesized hydantoin compounds, this study will also focus on the importance of specific ions involved in the biochemical and bioenergetic processes involved in active efflux, i.e., in calcium. The hydantoin group of compounds is known to be very effective in the modulation of ion-driven eukaryotic EPs and the calcium ion has a fundamental role in the inhibitory effect of hydantoin compounds on efflux pumps activity in eukaryotic cells. Therefore, to better understand its influence in the modulation of efflux activity in prokaryotic cells we proceeded to evaluate the accumulation/efflux of EtBr, using the model Enterobacteriaceae *Escherichia coli*, when in the presence of a calcium channel inhibitor – CPZ, and in the presence of a Ca²⁺ chelator – EDTA. By understanding the role of different ions in the biochemical and bioenergetics involved in active efflux, this will allow us to select more effective compounds when exposed to specific physiological conditions and a better understand of the biochemical conditions necessary for the efficient activity of the newly synthesized hydantoin compounds.

Studied the effect of calcium ions for bacterial transport channels and the activity of ATPases enzymes, the role of Ca^{2+} in the extrusion of efflux pump substrate EtBr, under challenging physiological conditions, will also be investigated.

Given the present concern over the lack of new antibiotics and the emergence of pathogens that have acquired resistance to multiple antibiotics causing infections that are effectively untreatable, there is an urgent necessity to identify clear targets to block efflux mechanisms and to design specific and more potent inhibitors with minimal adverse effects. Combination therapy which allows the administration of both antibacterial agents and EPIs may prevent the emergence of resistant Enterobacteriaceae and improve therapeutic treatment in patients suffering from these life-threatening infections.

III. MATERIAL AND METHODS

1. Assessment of the role of synthesized hydantoin derivates in various *Salmonella enterica* strains

1.1. Materials

1.1.1. Bacterial Strain

Wild-type strain *Salmonella enterica* serovar Enteritidis NCTC 13349 was kindly provided by Professor Seamus Fanning (School of Agriculture, Food Science, and Veterinary Medicine, UCD Veterinary Sciences Centre, University College Dublin, Dublin, Ireland).

Ciprofloxacin-resistant *Salmonella enterica* mutant strains 104-CIP and 5408-CIP, originated from *Salmonella enterica* serovar Enteritidis 104 and 5408 strains, were also provided by Professor Seamus Fanning (77). *Salmonella enterica* serovar Enteritidis 104 was exposed to increasing concentrations of ciprofloxacin (0.25, 0.5, 1, 2, 4, 8 and 16 mg/L) by conducting seven serial passages on tryptone soy agar (Oxoid, Hampshire, United Kingdom). The plate with the highest concentration that contained colonies was sub-cultured five times on antibiotic-free medium before antibiotic sensitivities were determined. The same protocol was conducted for *Salmonella enterica* serovar Enteritidis 5408 (77).

1.1.2. Culture media, compounds and solutions

The composition and preparation method of culture media and solutions used throughout the assays are described in Tables II and III.

Table II – Composition of culture media.

Culture Media	Composition (per liter of double distilled water)
Mueller-Hinton broth (MHB) ^(1, a)	300 g of dried beef infusion; 17.5 g of hydrolyzed casein; 1.5 g starch; 4.347 mg Ca ²⁺ ; 6.206 mg Mg ²⁺ , pH 7.3 ± 0.1 at 25°C

Mueller-Hinton MHB; 17 g of agar ⁽¹⁾; pH 7.3 ± 0.1 a 25°C
agar (MHA) ^(1, b)

⁽¹⁾ Oxoid Ltd., Basingstoke, England; ^(a) autoclaved at 121°C for 20 minutes at 1 bar in sterile test tubes and stored at 4°C; ^(b) autoclaved at 121°C for 20 minutes at 1 bar, distributed in sterile plates and stored at 4°C.

Table III – Composition and preparation method of the solutions used throughout the assays.

Stock Solutions	Composition and preparation
Phosphate buffered saline (PBS) 1X ^(1, a)	One tablet dissolved in 200 mL of double distilled water that contains 10 mM of phosphate buffer, 2.7 mM of potassium chloride and 137 mM of sodium chloride.
Glucose (Glu) ^(1,b)	0.2 g/mL in sterile double distilled water.

⁽¹⁾ Sigma Aldrich, St. Louis, USA; ^(a) autoclaved at 121°C for 20 minutes at 1 bar and stored at room temperature; ^(b) after preparing the solution, it was filtered with a polyvinylidene fluoride (PVDF) syringe filter (0.22µm pore size) from Rotilabo® (Spritzenfilter Steril, Karlshure, Germany) and stored at -20°C.

The composition and preparation method of antibiotic ciprofloxacin, EP inhibitor TZ and all hydantoin derivatives undergoing evaluation are described in Table IV.

Table IV – Composition and preparation method of antibiotic ciprofloxacin, EPI TZ and hydantoin derivatives.

Stock Solutions	Composition and preparation
Hydantoin derivates ^(a)	10 mg/mL in dimethyl sulfoxide (DMSO) ⁽¹⁾
Ciprofloxacin ^(2, b)	10 mg/mL in sterile double distilled water with addition of HCl ⁽¹⁾ until completely dissolved.
Thioridazine ^(3, b)	10 mg/mL in sterile double distilled water.

⁽¹⁾ Merck, Darmstadt, Germany; ⁽²⁾ Fluka Analytical, St. Louis, USA; ⁽³⁾ Sigma Aldrich, St. Louis, USA; ^(a) after preparation, all solutions were stored at -20°C; ^(b) after preparing the solution, it was filtered with a PVDF Syringe filter (0.22µm pore size) from Rotilabo® (Spritzenfilter Steril, Karlshure, Germany) and stored at -20°C.

All hydantoin derivatives were kindly provided by Dr. Jadwiga Handzlik and Prof. Dr. Katarzyna Kiec-Kononowicz from the Department of Technology and Biotechnology of Drugs, Medical College, Jagiellonian University, Cracow, Poland. These potential EPIs were evaluated for effects on the EP system of *Salmonella enterica* NCTC 13349.

Table V describes the composition and preparation method of universal EP substrate ethidium bromide (EtBr) used throughout the various fluorometric assays performed.

Table V – Composition and preparation method of substrate EtBr used by bacterial EPs.

Stock Solution	Composition and preparation
Ethidium bromide (EtBr) (1)	1 mg/mL in sterile double distilled water, from an initial stock concentration of 50 mg/ml, protected from light and stored at 4°C.

⁽¹⁾ Sigma Chemical Co., St. Louis, USA

1.2. Methods

1.2.1. Cultivation of bacterial strains *Salmonella* NCTC 13349 and ciprofloxacin-resistant *Salmonella* mutant's 104-CIP and 5408-CIP for MIC determinations

Wild-type strain *Salmonella enterica* serovar Enteritidis NCTC 13349 and ciprofloxacin-resistant *Salmonella enterica* serovar Enteritidis mutant's 104-CIP and 5408-CIP were cultivated in simple MHA, in MHA containing 4 mg/L of ciprofloxacin, and in MHA containing 16 mg/L of ciprofloxacin, respectively. All MH agar plates were incubated over-night at 37°C.

Afterward, isolated colonies from the MHA plates containing *Salmonella enterica* NCTC 13349, *Salmonella enterica* mutant 104-CIP and 5408-CIP were transferred to test tubes containing 5 mL of MH broth and MH broth supplemented with 4mg/L and 16 mg/L of ciprofloxacin, respectively. All test tubes were incubated over-night at 37°C in constant agitation (180 rpm) by the Thermo Scientific MaxQ 4000 (Iowa, USA).

Stocks from each one of the cultures in liquid medium were prepared in cryovials by adding 10% (v/v) glycerol (JT Baker, Philipsburg, USA) and kept at -80°C in the Nuaire Ultralow Freezer (Plymouth, Minnesota, USA).

1.2.2. Cultivation of bacterial strains for the fluorometric accumulation assays

Isolated colonies from the MHA plates containing *Salmonella enterica* NCTC 13349 and ciprofloxacin-resistant *Salmonella* mutant's 104-CIP and 5408-CIP (described in section 1.2.1.) were transferred to test tubes containing 10 mL of MH broth and grown at 37°C under constant agitation (180 rpm) (Thermo Scientific MaxQ 4000, Iowa, USA), until each culture reached an optical density (OD) of 0.6 transmittance at 600nm (Thermo Scientific SPECTRONIC 20D+ spectrophotometer, Fisher Scientific).

1.2.3. Determination of the MIC for *Salmonella enterica* NCTC 13349 reference strain in the presence of hydantoin derivatives, EtBr, TZ and DMSO

The minimum inhibitory concentration (MIC) is the primary test to determine the sensitivity of a bacterium to different compounds under study (100).

Before each MIC determination, fresh bacterial strains of *Salmonella enterica* NCTC 13349 were prepared according to protocol described in section 1.2.1.

Table VI indicates the concentration of the stock solutions and the concentration range used to determine the MIC value for all hydantoin compounds under study, for substrate EtBr and for the control EPI TZ. The MIC was defined as the lowest concentration of compound for which no bacterial growth was observed (79).

Concerning the solvent used, instead of setting a range of concentrations, we tested the exact amount of DMSO that would be used in all fluorometric assays, and thus verify directly if the percentage employed would inhibit bacterial growth.

Table VI – Range of concentrations used in determining the MIC for hydantoin compounds, for EtBr and for TZ. Evaluation of the effect of 10% of DMSO.

Bacterial Strain	Compound	Concentration of the Stock Solution	Concentration Range
<i>Salmonella enterica</i> NCTC 13349	Hydantoin derivatives	10 mg/mL	240 – 0.469 mg/L
	EtBr	10 mg/mL	256 – 0.5 mg/L
	DMSO	100%	10%
	TZ	10 mg/ml	256 – 0.5 mg/L

The MICs for all compounds undergoing evaluation, i.e., the hydantoin derivatives, substrate EtBr and phenothiazine TZ were determined by the broth microdilution method in 96-well microtitre plates according to the *Clinical and Laboratory Standards Institute* (CLSI) guidelines (113).

In this MIC determination four test controls were performed: (a) MHB sterility test, (b) bacterial growth control (MHB and the bacterial strain under study), in order to evaluate bacterial viability, (c) solvent control (MHB containing solvent at the same concentration as the compounds, and the bacterial strain), to ensure that the solvent has no inhibitory effect on bacterial growth, and (d) compound control (MHB and compound) to assess turbidity that may interfere in the reading of results.

From the test tube containing *Salmonella enterica* NCTC 13349 that was placed in an over-night growth (described in section 1.2.1.), a bacterial suspension was prepared in 10 mL of PBS 1X by adjusting turbidity to 0.5 in the McFarland scale. Aliquots of 0.02 mL of bacterial suspension were transferred to each well of the 96-well microtitre plate (with the exception of the well used for the sterility test and compound control) that contained each compound at different concentrations obtained by serial dilutions in MHB. The microplates were incubated at 37°C and the MICs results observed after 16 and 18 hours.

For the DMSO measurement, the solvent was added to a well containing MHB and 0.02 mL of the bacterial suspension in order to obtain a final percentage of 10%. This percentage corresponds to the ratio used in fluorometric assays.

After the incubation period, each assay was validated by the presence of growth in the well used for the bacterial growth control (positive control) and no growth in the well used for the MHB sterility test (negative control). The other controls were utilized for comparison purposes, facilitating the reading of the results. All MICs were performed in triplicate for each compound to ensure that the results obtained were consistent.

In this assay it is important to emphasize some key points. It is essential to monitor and evaluate carefully the solvent control, since it may be responsible for inhibiting bacterial growth and lead to inaccurate observations that influence the MIC values of the hydantoin compounds.

In order to assure that the inhibitory effect of the compounds against active efflux by *Salmonella enterica* did not compromise cellular viability and the growth rate of the bacterium, the concentrations used in the following work did not exceed half the MIC value (53, 79).

1.2.4. Fluorometric assay performed by the Real-Time Thermal Cycler Rotor-GeneTM 3000

After determining the MIC values, all compounds were tested in the Real-Time Thermal Cycler Rotor-GeneTM 3000 to observe their behavior in the presence of universal efflux substrate EtBr and glucose as the immediate source of energy. To quantify the amount of relative fluorescence emitted by EP substrate EtBr when accumulated in a specific bacterial strain, a semi-automated fluorometric method, standardized in the Group of Micobacteria of the Unit of Microbiology of the IHMT/UNL, was utilized.

As mentioned earlier, this method which employs the Rotor-GeneTM 3000 thermo cycler (Corbett Research, Australia) and uses EtBr as the fluorescent probe, which emits a weak fluorescence in aqueous solution, but becomes strongly fluorescent within the periplasm. This occurs due to its ability to bind to cellular components, making it

possible to distinguish accumulation from efflux itself (79), under varying physiological conditions, such as pH, presence/absence of an energy source and presence of EPIs (108). The instrument also allows the selection of the excitation and emission wavelengths that for EtBr are 530 nm band-pass (bp) and 585 nm high-pass (hp) filters, respectively (79).

1.2.4.1. EtBr accumulation assay to assess the effect of hydantoin derivatives in *Salmonella enterica* NCTC 13349

Bacterial growth. As previously elucidated in section 1.2.2., *Salmonella enterica* NCTC 13349 was grown in MHB until it reached an optical density (OD) of 0.6 at 600nm of transmittance, which corresponds to the exponential growth phase (i.e., mid-log phase), measured by the Thermo Scientific SPECTRONIC 20D+ spectrophotometer (Fisher Scientific).

Cells were collected in aliquots of 1 mL and centrifuged at 13.000 rpm for 3 minutes at room temperature in a mini-centrifuge (Biofuge Pico Heraeus, Kendro Laboratory Products, Osterode, Germany). They were washed in PBS 1X pH 7.4 (preparation described in Table III), the pellet re-suspended and centrifuged again. This process was repeated twice. Then, by adding the same buffer, the OD was adjusted to 0.6 transmittance at 600nm by the PU8620 UV/VIS/NIR spectrophotometer (Philips, Cambridge, United Kingdom), allowing the assay to run with a 0.3 absorbance value which corresponds to the optimal cellular concentration set to run in the Rotor-GeneTM 3000 thermo cycler.

Determination of the optimal concentration of substrate EtBr. To determine the optimal concentration of EtBr that could be utilized in the fluorometric assays that follow, in order to evaluate the behavior of the hydantoin compounds, a single assay was conducted where *Salmonella enterica* NCTC 13349 strain was subjected to the presence of increasing concentrations of EtBr with and without glucose.

For this, various “work solutions” of 1 mL containing EtBr (Table V) to yield final concentrations of 0.5, 1, 2, 3, and 4 mg/L with and without glucose (Table III) at 0.4% were prepared. The volume was adjusted with the addition of PBS 1X at pH 7.4.

After preparing all solutions and adjusting the cellular OD to 0.6 at 600nm of transmittance, aliquots of 0.05 mL of each “work solution” were transferred to 0.2 mL tubes containing 0.05 mL of bacterial cells in PBS 1X pH 7.4. They were incubated in the thermocycler for 60 cycles at 37°C to quantify the amount of relative fluorescence emitted by different concentrations of EtBr in *Salmonella enterica* NCTC 13349.

Solution preparation. A “work solution” of 1 mL containing EtBr (Table V) and glucose (Table III) was prepared in order to counter final concentrations of 1 mg/L and 0.4%, respectively. The volume was adjusted with the addition of PBS 1X at pH 7.4.

Hydantoin compounds were prepared separately, allowing us to control and limit the amount of DMSO in each test tube. From the 10 mg/mL stock solutions in DMSO (Dimethyl sulfoxide) (Table IV), intermediate solutions were prepared in order to yield final concentrations of 120 mg/L and 80 mg/L in the RotorGene test tubes.

After preparing all solutions and adjusting the cellular OD, aliquots of 0.045 mL of the “work solution” and 0.01 mL of the compound intermediate solutions were transferred to 0.2 mL tubes containing 0.045 mL of bacterial cells in PBS 1X pH 7.4. They were incubated in the thermocycler for 60 cycles at 37°C to quantify the amount of relative fluorescence emitted by EtBr when it accumulates in *Salmonella enterica* NCTC 13349.

Once again, several controls were prepared and run in parallel with the compounds under study. Each assay contained the following controls: EtBr plus compound (1mg/L of EtBr and hydantoin compound), solvent control (1mg/L of EtBr, 0.4% of glucose, bacterial strain and DMSO), EtBr control (1mg/L of EtBr, 0.4% of glucose and bacterial strain). The EtBr plus compound control is further explained in section 1.2.4.2., which corresponds to the auto-fluorescence assays.

From the real-time data, the activity of the compound, namely the relative final fluorescence (**RFF**) of the last time point (minute 60) of the EtBr accumulation assay was calculated according to the following formula, described by Ramalhete et al (94):

$$\mathbf{RFF} = \frac{\mathbf{RF}_{\text{treated}} - \mathbf{RF}_{\text{untreated}}}{\mathbf{RF}_{\text{untreated}}}$$

The $\mathbf{RF}_{\text{treated}}$ value is equivalent to the relative fluorescence (RF) at the last time point of EtBr retention curve in the presence of an inhibitor. The $\mathbf{RF}_{\text{untreated}}$ value refers to the relative fluorescence at the last time point of EtBr retention curve of the untreated control having the solvent control (DMSO). As shown in Figure 6, the value obtained at point 60 in the presence and absence of an EPI is utilized in order to calculate the value of RFF.

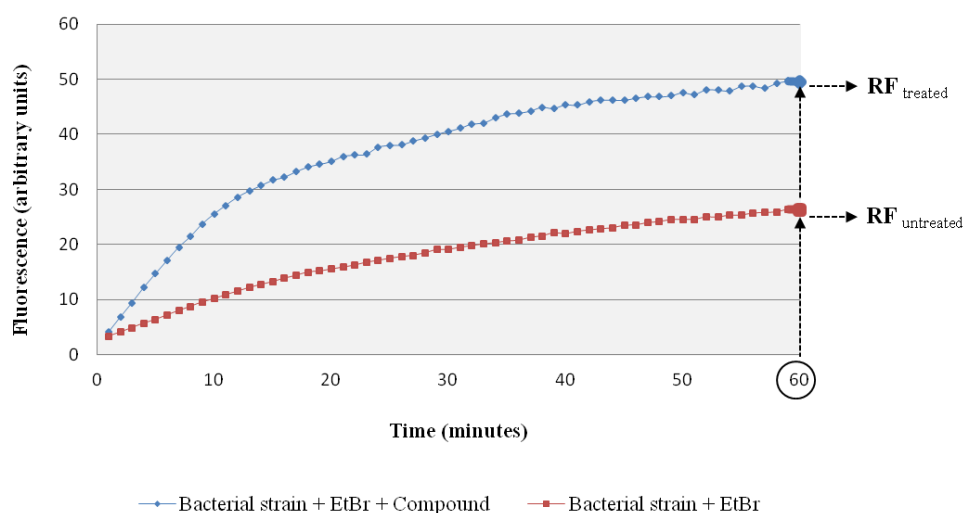


Figure 6 – Schematic representation of the fluorescence values selected to calculate the RFF value. The blue point represents the fluorescence value obtained after 60 minutes in the presence of a potential EPI, whereas the red point represents fluorescence value obtained after 60 minutes in the absence of EPI, i.e., the control assay.

The greater the difference between $\mathbf{RF}_{\text{treated}}$ and $\mathbf{RF}_{\text{untreated}}$, the greater the degree of EtBr accumulation and therefore, the greater the degree of inhibition of the EP system of the bacterium promoted by the agent and its concentration.

All experiments were repeated three times and the RFF values presented are the average of three independent assays.

1.2.4.2. Auto-fluorescence assay

In respect to the hydantoin derivatives, the only information we possess is their molecular weight and chemical formula. Therefore, we evaluated all the compounds for possible self-fluorescence properties that could interfere in the fluorescence assays.

Thus, 0.01 mL of the intermediate solutions of each hydantoin compound were transferred to 0.2 mL tubes containing 0.09 mL of PBS 1X plus EtBr at 1mg/L. This was performed for both concentrations tested in each compound. All 0.2 mL tubes were placed in RotorGene and testing was done under the same conditions previously described. The auto-fluorescence values were subtracted from the values obtained in the EtBr accumulation assays performed by *Salmonella* NCTC 13349, outlined above.

1.2.5. Determination of MIC for two ciprofloxacin-resistant *Salmonella* mutant strains in the presence of hydantoin compound SZ-7, ciprofloxacin and TZ

Hydantoin compounds that presented cumulative effects in the fluorometric assays (described in section 1.2.4.1) were submitted to a new MIC determination, but this time in the presence of ciprofloxacin using ciprofloxacin-resistant *Salmonella* 104-CIP and 5408-CIP mutant strains.

The MIC determination between SZ-7 and ciprofloxacin allows us to verify if the compound is capable of reducing the MIC of ciprofloxacin in both *Salmonella* mutants strains. By comparing the MIC of ciprofloxacin plus SZ-7 with the MIC of ciprofloxacin alone we can verify if the compound possesses a synergistic effect when associated with ciprofloxacin.

The MIC value for TZ was also determined for both ciprofloxacin-resistant strains, in the presence and absence of ciprofloxacin. This determination will make it possible to compare the hydantoin compound to TZ, a very well known and studied EPI.

For these assays, quinolone-resistant mutant's *Salmonella* 104-CIP and 5408-CIP were cultivated in MHA and MHB, according to the protocol described in section 1.2.1. All of the following assays were performed using the broth microdilution method in 96-well

microtitre plates according to the *Clinical and Laboratory Standards Institute* (CLSI) guidelines (113).

After performing point 1.2.1., separate MICs determinations were done for ciprofloxacin, SZ-7 (hydantoin compound) and phenothiazine TZ by *Salmonella* 104-CIP and 5408-CIP. The protocol used for these assays is similar to the one previously described in section 1.2.3. Below, Table VII indicates the range of concentrations used for each compound to determine the respective MIC value.

Table VII – Range of concentrations used for the MIC determinations of ciprofloxacin, hydantoin derivative SZ-7 and phenothiazine TZ.

Bacterial Strain	Compounds	Concentration of the Stock Solution	Concentration Range
<i>Salmonella enterica</i> 104-CIP and 5408-CIP	ciprofloxacin	10 mg/mL	240 – 0.469 mg/L
	SZ-7 (hydantoin derivative)	10 mg/mL	240 – 0.469 mg/L
	TZ	10 mg/mL	256 – 0.5 mg/L

Subsequently, the MIC values for both *Salmonella* 104-CIP and 5408-CIP in the presence of both ciprofloxacin and SZ-7 were evaluated. This time, the range of concentrations used for ciprofloxacin was between 256 – 0.5 mg/L, while SZ-7 yielded final concentrations of 50 and 100 mg/L.

Again, in 10 mL of PBS 1X solution the bacterial cultures turbidity was adjusted to 0.5 McFarland scale. Aliquots of 0.02 mL of bacterial suspension and 0.005 mL of SZ-7 were transferred to each well of the 96-well microtitre plate that contained ciprofloxacin distributed by serial dilutions in MHB, in order to respect the range of concentrations above referenced. The microplate was incubated at 37°C and the MIC results observed after 16 and 18 hours. Regarding to the amount of SZ-7, only 0.005 mL were

transferred to avoid precipitation and to decrease the amount of solvent capable of inhibiting bacterial growth, while maintaining concentrations of 50 and 100 mg/L.

For these determinations, the controls used and the validation method are equivalent to those described previously in section 1.2.3.

1.2.6. Fluorometric assay to assess the effect of SZ-7 on the accumulation of EtBr in two ciprofloxacin-resistant *Salmonella* mutant strains

After determining the MIC values, hydantoin derivative SZ-7 and phenothiazine TZ were tested in the Real-Time Thermal Cycler Rotor-GeneTM 3000 to examine their behavior in the presence of universal efflux substrate EtBr and glucose as the source of metabolic energy, by two ciprofloxacin-resistant *Salmonella* mutant strains.

1.2.6.1. Accumulation protocol of EP substrate EtBr

Bacterial growth. As pointed out in section 1.2.2., *Salmonella* 104-CIP and 5408-CIP were grown in MHB until they reached an optical density (OD) of 0.6 at 600nm of transmittance (Thermo Scientific SPECTRONIC 20D+ spectrophotometer, Fisher Scientific).

Cells were collected in aliquots of 1 mL and centrifuged at 13.000 rpm for 3 minutes at room temperature in a mini-centrifuge (Biofuge Pico Heraeus, Kendro Laboratory Products, Osterode, Germany). Cells were washed in PBS 1X pH 7.4, the pellet re-suspended and centrifuged again. This process was repeated twice. Then, the OD was adjusted to 0.6 transmittance at 600nm (PU8620 UV/VIS/NIR spectrophotometer, Philips, Cambridge, United Kingdom) using the same buffer.

Determination of the optimal concentration of substrate EtBr. A single fluorometric assay was performed using two ciprofloxacin-resistant *Salmonella* mutant strains in the presence of various concentrations of EtBr, in order to select the substrate's optimal concentration of accumulation and efflux. The protocol utilized is analogous to the one elucidated in section 1.2.4.1.

Solution preparation. Different “work solutions” of 1 mL were prepared: a tube with EtBr and a tube with EtBr and glucose. EtBr and glucose were prepared in order to counter final concentrations of 1 mg/L and 0.4%, respectively. Final volumes were adjusted by adding PBS 1X at pH 7.4.

Again, hydantoin derivative SZ-7 was prepared separately, allowing us to control and limit the amount of DMSO in the test tube. From the 10 mg/mL stock solutions, intermediate solutions of the derivative were prepared in order to yield final concentrations of 120 mg/L and 80 mg/L.

To maintain consistency among the assays, TZ was also prepared separately. Using a stock solution of 10 mg/ml, an intermediate solution was prepared utilizing bi-distilled sterile water, in order to achieve final concentrations of 64 and 128 mg/L.

After preparing all solutions and adjusting the cellular OD, aliquots of 0.045 mL of the “work solutions” and 0.01 mL of the compound intermediate solutions (whether it be SZ-7 or TZ) were added to 0.2 mL tubes containing 0.045 mL of cells in PBS 1X pH 7.4. They were incubated in the thermocycler for 60 cycles at 37°C to quantify the amount of relative fluorescence emitted by EtBr when accumulated in *Salmonella* 104-CIP and 5408-CIP mutant strains.

Several controls were prepared and run in parallel with the compounds under study. Each assay contained the following controls: EtBr control (1mg/L of EtBr with and without 0.4% of glucose and bacterial strain), and EtBr plus compound control (1mg/L of EtBr, 0.4% of glucose and hydantoin compound, i.e., without the bacterial strain).

2. Assessment of the role of calcium in *E. coli* reference strain, in the presence of chlorpromazine and ethylenediaminetetraacetic acid.

2.1. Materials

2.1.1. Bacterial Strain

Wild-type strain *E. coli* AG100 (K-12 *argE3 thi-1 rpsL xyl mtl* $\Delta(gal-uvrB)$ *supE44*), which contains an AcrAB-TolC efflux system intact and functional (74).

2.1.2. Culture media, compounds and solutions

The composition and preparation method of culture media, compounds and solutions used throughout the assay are described in Tables VIII to X. However, the composition and preparation method of culture media MHB and MHA, and universal EP substrate EtBr were previously described in Tables II and V, respectively.

Table VIII – Composition of culture media.

Culture Media	Composition (per liter of double distilled water)
Luria Bertani (LB) ^(a)	10 g casein peptone ⁽¹⁾ ; 5 g yeast extract ⁽¹⁾ ; 10 g sodium chloride (NaCl) ⁽²⁾
Luria Bertani agar (LA) ^(b)	LB; 20 g of agar ⁽³⁾

⁽¹⁾ Merck, Darmstadt, Germany; ⁽²⁾ Panreac Química, Barcelona, Spain; ⁽³⁾ Oxoid Ltd., Basingstoke, England; ^(a) autoclaved at 121°C for 20 minutes at 1 bar in sterile test tubes and stored at 4°C; ^(b) autoclaved at 121°C for 20 minutes at 1 bar, distributed in sterile plates and stored at 4°C.

Table IX – Composition and preparation method of the solutions.

Stock Solutions	Composition and preparation
PBS 1X, pH 8 ^(1, a)	One tablet dissolved in 200 mL of double distilled water that contains 10 mM of phosphate buffer, 2.7 mM of potassium

	chloride and 137 mM of sodium chloride.
	The pH of 1X PBS at pH 7.4 was adjusted to pH 8 by adding a solution of sodium hydroxide (NaOH) ⁽²⁾ . Measurements were performed by the Metrohm pH-Meter 632 (Herisan, Switzerland).
Glucose ^(1,b)	0.2 g/mL in double distilled sterile water.
Calcium Chloride ^(2, b) (CaCl₂)	50 mM and 100 mM in double distilled sterile water

⁽¹⁾ Sigma Aldrich, St. Louis, USA; ⁽²⁾ Merck, Darmstadt, Germany; ^(a) autoclaved at 121°C for 20 minutes at 1 bar and stored at room temperature; ^(b) after preparing the solution, it was filtered with a PVDF syringe filter (0.22µm pore size) from Rotilabo® (Spritzenfilter Steril, Karlsruhe, Germany) and stored at -20°C.

Table X – Composition and preparation method of ATPase inhibitor CPZ and Ca²⁺ chelator EDTA.

Stock Solutions	Composition and preparation
Chlorpromazine (CPZ) ^(1,a)	10 mg/mL and 1mg/mL in double distilled sterile water
Ethylenediaminetetraacetic acid (EDTA) ^(1, b)	Prepared in double distilled water, under constant agitation with addition of NaOH ⁽²⁾ until completely dissolved (around pH 8)

⁽¹⁾ Sigma Aldrich, St. Louis, USA; ⁽²⁾ Merck, Darmstadt, Germany; ^(a) after preparing the solution, it was filtered with a PVDF syringe filter (0.22µm pore size) from Rotilabo® (Spritzenfilter Steril, Karlsruhe, Germany) and stored at -20°C; ^(b) autoclaved at 121°C for 20 minutes at 1 bar and stored at room temperature.

2.2. Methods

2.2.1. Cultivation of bacterial strain *E. coli* AG100 for MIC determinations

Wild-type strain *E. coli* AG100 was cultivated in MHA plates and incubated over-night at 37°C. Isolated colonies were then transferred to 5 mL of MHB and incubated over-night at 37°C under constant agitation (180 rpm) by the Thermo Scientific MaxQ 4000

(Iowa, USA). Stocks of the cultures were made according to the protocol elucidated in section 1.2.1.

2.2.2. Cultivation of bacterial strain *E. coli* AG100 for the fluorometric accumulation and efflux assays

Wild-type *E. coli* K-12 AG100 strain was cultivated in LA (incubated over-night at 37°C) and isolated colonies were transferred to 10 mL of LB (incubated at 37°C under constant agitation by the Thermo Scientific MaxQ 4000 (Iowa, USA)). Again, stocks of the cultures obtained in liquid medium were prepared according to the protocol described in section 1.2.1.

2.2.3. Determination of MIC for *E. coli* AG100 reference strain in the presence of CPZ, EDTA and EtBr

Before each MIC determination, fresh bacterial strains were prepared in accordance with protocol described in section 2.2.1.

Below, Table XI indicates the concentration of the stock solutions and the concentration range used to determine the MIC values for Ca²⁺ channel inhibitor CPZ, Ca²⁺ chelator EDTA and efflux substrate EtBr. Again, the MIC was defined as the lowest concentration in which no bacterial growth was observed (79).

Table XI – Range of concentrations used for the MIC determination of CPZ, EDTA and EtBr.

Bacterial Strain	Compound	Concentration of the Stock Solution	Concentration Range
<i>E. coli</i> AG100	CPZ	10 mg/mL	320 – 0.63 mg/L
	EDTA	0.5 M	160 – 0.31 mM
	EtBr	10 mg/mL	300 – 0.59 mg/L

The MICs were determined by the broth microdilution method in 96-well microtitre plates according to the *Clinical and Laboratory Standards Institute* (CLSI) guidelines (113). In every MIC assay four test controls were made, as explained previously in section 1.2.3.

Briefly, a bacterial suspension was prepared by adjusting the turbidity to 0.5 according to the McFarland scale in PBS 1X pH 8. Aliquots of 0.02 mL of bacterial suspension were transferred to each well of the 96-well microtitre plate (with the exception of the well used for the sterility test and compound control) that contained each compound at different concentrations obtained by serial dilutions (MH broth and compound). The microplates were incubated at 37°C and the MICs results observed after 16 and 18 hours.

The validation and control points are equivalent to the ones used in section 1.2.3.

2.2.4. Fluorometric assay performed by the Real-Time Thermal Cycler Rotor-GeneTM 3000

After determining the MIC values for CPZ, EDTA and EtBr, these compounds were analyzed with the aid of the Real-Time Thermal Cycler Rotor-GeneTM 3000 to observe their behavior in the presence of varying concentrations of calcium, of glucose as the immediate source of energy, and at pH 8.

2.2.4.1. Accumulation protocol of EP substrate EtBr

Bacterial growth. As pointed out in section 2.2.2, *E. coli* AG100 was grown in LB until it achieved an OD of 0.6 at 600 nm of transmittance (Thermo Scientific SPECTRONIC 20D+ spectrophotometer, Fisher Scientific).

Cells, collected in aliquots of 1mL, were washed, re-suspended and centrifuged for 3 minutes at 13,000 rpm (Heraeus Biofuge Pico, Osterode, Germany). This process was repeated twice in PBS 1X pH 8 (preparation method described in Table IX). Then, by adding PBS 1X pH 8, the OD was adjusted to 0.6 at 600nm (PU8620 UV/VIS/NIR

spectrophotometer, Philips, Cambridge, United Kingdom), allowing the assay to run with a 0.3 absorbance value.

Determination of the optimal concentration of substrate EtBr. A single fluorometric assay was performed using *E. coli* AG100 reference strain in the presence of various concentrations of EtBr, in order to select the substrate's optimal concentration of accumulation and efflux. The protocol utilized is analogous to the one elucidated in section 1.2.4.1.

Solution preparation. All solutions were prepared in eppendorfs with a final volume of 1mL. Then, 0.05 mL of each solution was transferred to 0.2 mL microtubes, followed by the addition of 0.05 mL of the bacterial suspension previously washed and adjusted to 0.6 at 600 nm of transmittance.

For each test tube different experimental conditions and controls were prepared, as described below.

- 0.05 mL of a solution containing PBS 1X pH 8 and EtBr at 2 mg/L; and 0.05 mL of PBS at pH 8
- 0.05 mL of a solution containing PBS 1X pH 8, 0.8% of glucose and EtBr at 2 mg/L; and 0.05 mL of PBS 1X pH 8
- 0.05 mL of the bacterial suspension; and 0.05 mL of PBS 1X pH 8;
- 0.05 mL of the bacterial suspension; and 0.05 mL of a solution containing PBS 1X pH 8, and 0.8% of glucose;
- 0.05 mL of the bacterial suspension; and 0.05 mL of a solution containing PBS 1X pH 8, 0.8% of glucose and EtBr at 2 mg/L;
- 5 different solutions: 0.05 mL of the bacterial suspension; and 0.05 mL containing PBS 1X pH 8, 0.8% of glucose, EtBr at 2 mg/L and CPZ to yield 20, 40, 60, 80 and 100 mg/L.

- 6 different solutions: 0.05 mL of the bacterial suspension; and 0.05 mL containing PBS 1X pH 8, 0.8% of glucose, EtBr at 2 mg/L and calcium chloride to yield 2, 4, 6, 8, 10 and 20 mM.
- 6 different solutions: 0.05 mL of the bacterial suspension; and 0.05 mL containing PBS 1X pH 8, 0.8% of glucose, EtBr at 2 mg/L, CPZ at 40 mg/L and calcium chloride to yield 2, 4, 6, 8, 10 and 20 mM.
- 6 different solutions: 0.05 mL of the bacterial suspension; and 0.05 mL containing PBS 1X pH 8, 0.8% of glucose, EtBr at 2 mg/L and EDTA to yield 2, 4, 6, 8, 10 and 20 mM.
- 6 different solutions: 0.05 mL of the bacterial suspension; and 0.05 mL containing PBS 1X pH 8, 0.8% of glucose, EtBr at 2 mg/L, EDTA at 10 mM and calcium chloride to yield 2, 4, 6, 8, 10 and 20 mM.
- 6 different solutions: 0.05 mL of the bacterial suspension; and 0.05 mL containing PBS 1X pH 8, 0.8% of glucose, EtBr at 2 mg/L, EDTA at 20 mM, and calcium chloride to yield 2, 4, 6, 8, 10 and 20 mM.

In the Corbett 3000 thermocycler, aliquots with a final volume of 0.1 mL were tested to assess the effect of CPZ and EDTA on the accumulation of EtBr with and without the modulation effect of Ca^{2+} . All test tubes were incubated in the thermocycler at 37°C and fluorescence was measured over a period of 60 cycles (approximately 60 minutes).

To note, all concentrations in the test tubes correspond to half the concentration in the solutions initially prepared. In other words, the test tubes contained EtBr and glucose to yield final concentrations of 1 mg/L and 0.4%, respectively, with varying concentrations of CPZ (10, 20, 30, 40 and 50 mg/L) with and without varying concentrations of Ca^{2+} (1, 2, 3, 4, 5 and 10 mM), and, varying concentrations of EDTA (1, 2, 3, 4, 5 and 10 mM) with and without varying concentrations of Ca^{2+} (1, 2, 3, 4, 5 and 10 mM).

2.2.4.2. Efflux protocol of EP substrate EtBr

Protocols regarding bacterial growth and the determination of the optimal concentration of substrate EtBr are similar to the ones described in section 2.2.4.1.

Solution preparation. For this assay, we used the solutions previously prepared in eppendorfs with a final volume of 1 mL. Initially, the fluorescence in microtubes containing 0.045 mL of bacterial cells in PBS pH 8, no glucose and EtBr to yield a final concentration of 1 mg/L was followed for approximately 40 minutes at 37°C. The instrument was paused and 0.045 mL of various solutions undergoing evaluation were added, namely: PBS pH 8 alone, PBS pH 8 containing glucose to yield a final concentration of 0.4%, PBS pH 8 containing 0.4% of glucose with varying concentrations of CPZ, PBS pH 8 containing 0.4% of glucose with varying concentrations of CPZ in combination with varying concentrations of Ca^{2+} , PBS pH 8 containing 0.4% of glucose with varying concentrations of EDTA, PBS pH 8 containing 0.4% of glucose with varying concentrations of EDTA in combination with varying concentrations of Ca^{2+} . Immediately after adding the solutions, the instrument was restarted and the fluorescence values were followed for an additional period of 10 minutes.

IV. RESULTS

1. Assessment of the role of newly synthesized hydantoin derivates in various *Salmonella enterica* strains

1.1. MIC values obtained for the hydantoin compounds, EtBr and TZ by *Salmonella enterica* NCTC 13349 reference strain

To be able to evaluate the activity of any agent against EP systems, the concentrations tested must correspond to concentrations that do not affect cellular viability and replication (108).

Tables XII and XIII present the MIC values obtained for every hydantoin compound under study, for universal efflux substrate EtBr and for EPI thioridazine (TZ) which is used as a positive control. Also, outlined below is the result obtained when *Salmonella enterica* serovar Enteritidis NCTC 13349 was submitted to the presence of 10% of DMSO, in order to evaluate cellular viability. All assays were repeated three times, in order to ensure reproducibility and consistency of results.

Table XII - MIC values obtained by *Salmonella enterica* NCTC 13349 for substrate EtBr and for TZ. Also, the result obtained from the assay used to evidence bacterial growth in the presence of DMSO at 10%.

<i>Salmonella</i> NCTC	DMSO at 10%	MIC for EtBr (mg/L)	MIC for TZ (mg/L)
13349	growth with a slight inhibition	128	128

Table XIII – MIC values obtained for all hydantoin derivatives under study by *Salmonella enterica* NCTC 13349.

Synthesized Compounds	MIC (mg/L)
(Halogen)phenylpiperazine derivatives of 5-phenylhydantoin (modifications of BS-1): GG-2a ⁽²⁾ , GG-4a ⁽²⁾ , MN-1, MN-2 ⁽²⁾ , MN-4 ⁽²⁾ , MN-5 ⁽²⁾ , MN-6, BS-5, BS-6, TN-2 ⁽²⁾ , TN-3 ⁽²⁾ , BG-1 ⁽²⁾ , BG-2 ^(1, 2) and JH-1 ⁽²⁾	>240
N3-alkylamine derivatives of 5,5-diphenylhydantoin (modifications of KF-2): M-1, M-2, M-3, M-4, Ksc-5 and Ksc-7	> 240
Halogenphenylpiperazine derivatives of 5-arylidenehydantoin (modifications of AD-26): AD-38 ⁽²⁾ , AD-39, AD-40 ⁽²⁾ , AD-42, AD-43, AD-44, AD-45, AD-46 and AD-49	> 240
5-Alkoxyarylidenehydantoin (modification of SZ-7): HY-42, HY-43, HY-44, HY-46, HY-47, HY-72, HY-73, AM-1, AM-2, AM-3 and AM-4	> 240
Other hydantoin derivatives: SZ-2, SZ-7 ⁽²⁾ , LL-9, BS-1, JH-63, MN-3, TD-7k, GG-5k, P3, P7, RW-15b, AD-26, RW-13, AD-29, KF-2, PDPH-3, Mor-1, XV, Thioam-1, JHF-1, JHC-2, JHP-1 and Fur-2	> 240

Legend: ⁽¹⁾ Heated to dissolve completely; ⁽²⁾ presented precipitation in the microtitre plate, complicating the reading of the results.

As shown in Table XIII, due to the fact that the MIC values of all sixty-three hydantoin derivatives exceeded 240 mg/L, the sub-inhibitory concentrations employed in the fluorometric assays that follow were 80 and 120 mg/L.

The MIC value obtained by *Salmonella enterica* serovar Enteritidis NCTC 13349 for EtBr and TZ, presented in Table XII, also provides information as to what sub-inhibitory concentrations can be used in the semi-automatic fluorometric assays that follow. TZ, as is the case of the hydantoin compounds, was used at a quarter and at half of the MIC value to guarantee no interference in cellular viability.

In relation to EtBr, the optimal concentration to use in the fluorometry assays corresponds to the concentration at which minimum accumulation is observed. To select this concentration, an accumulation assay with *Salmonella* NCTC 13349 was conducted utilizing substrate EtBr at varying concentrations in the presence and absence of glucose as an immediate energy source (as shown in Figure 7).

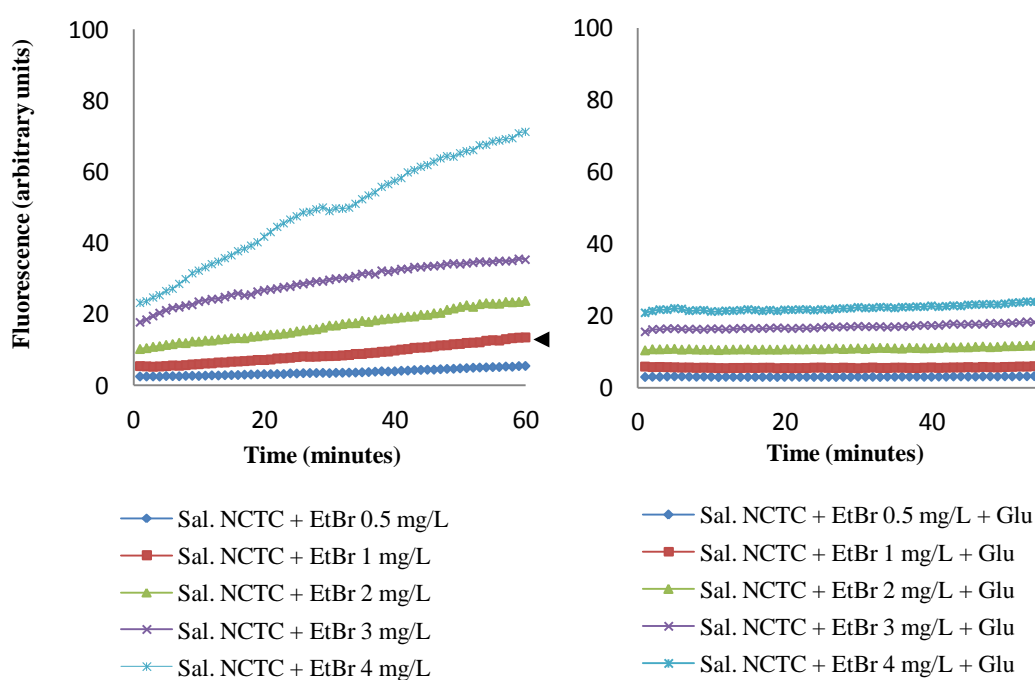


Figure 7 – Accumulation of EtBr at varying concentrations by *Salmonella enterica* NCTC 13349. Accumulation of EtBr to yield final concentrations of 0.5, 1, 2, 3, and 4 mg/L in the absence and presence of glucose at 0.4%.

The selected concentration of EtBr was 1 mg/L, since in both assays *Salmonella* 13349 presented values of fluorescence below 10% with a slight tendency to accumulate. By using this concentration of substrate simultaneously with inhibitory compounds undergoing evaluation, if an inhibition of efflux occurs we will be able to observe it

since the device is capable of detecting the substrates signal. If we were to use higher concentrations of substrate EtBr in the presence of the EPI under study, the fluorescence values obtained wouldn't be detected by the device since the fluorescence readings are limited.

The selection of the optimal concentration of EtBr to use in the fluorometric assays for the remaining strains were chosen based on the same criteria elucidated above.

1.2. Accumulation of EtBr in the presence of various hydantoin derivates in *Salmonella enterica* NCTC 13349 by the fluorometric assay

Sixty-three hydantoin compounds were evaluated for their efflux modulating effects in the intrinsic efflux activity of *Salmonella enterica* serovar Enteritidis NCTC 13349 by using real-time fluorometry. This method that uses fluorescent substrate EtBr provides a powerful tool to continuously monitor transport, since EtBr penetrates the bacterial cell wall and accumulates in the periplasmic space of Gram-negative bacteria when its concentration exceeds the capacity of the EP system (108) or its extrusion by the EP system is impaired by an inhibitory agent (74, 107).

To control and monitor whether hydantoin compounds possess inhibitory effects on EP system of *Salmonella* NCTC strain, an assay was carried out in parallel using TZ as the EPI. Given the information regarding this compound and its inhibitory properties on several strains, it was used for comparison purposes as a positive control.

In Figure 8 we can observe the effect that TZ possesses on the accumulation of EtBr by *Salmonella enterica* serovar Enteritidis NCTC 13349, in the presence of metabolic energy.

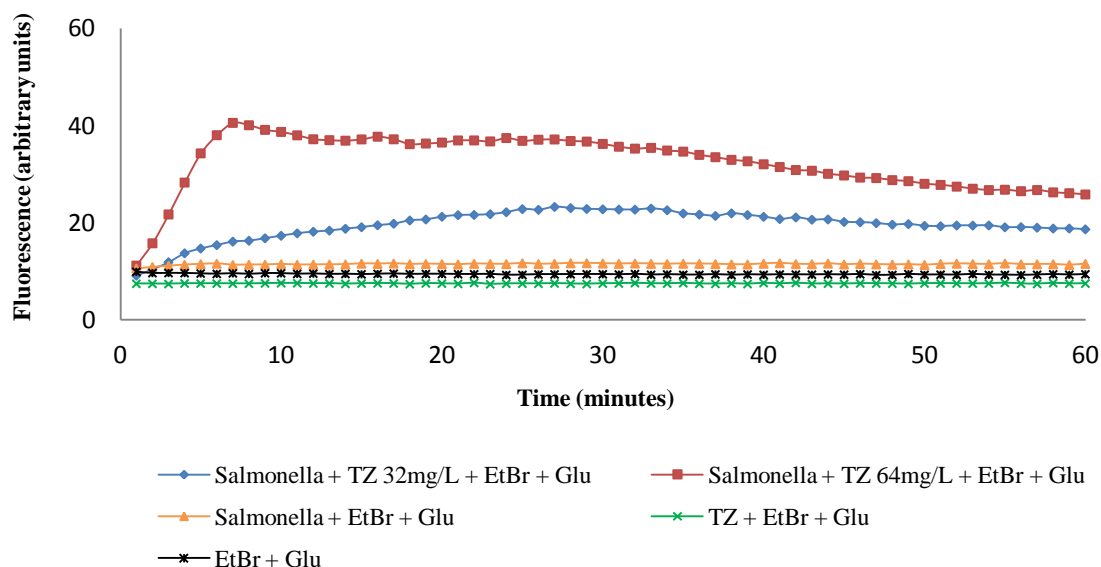


Figure 8 – Accumulation of EtBr by *Salmonella enterica* NCTC 13349 in the presence of varying concentrations of TZ. Accumulation of EtBr at 1mg/L in the presence of glucose at 0.4% and TZ to yield final concentrations of 32 and 64 mg/L.

As shown in Figure 8, TZ at 64 mg/L (1/2 MIC value) presents a somewhat elevated accumulation rate in the beginning of the assay until a gradual steady-state is reached and, over time the steady-state between accumulation and efflux of EtBr starts to decrease moderately. This data shows that the strain involved, despite enduring the inhibitory effects of the EPI at the beginning of the assay, quickly starts to efflux the compound that accumulates up to a level that can jeopardize its survival.

1.2.1. Auto-fluorescence assays obtained by the hydantoin derivatives

Due to the lack of information of the hydantoin derivatives under study, we previously confirmed by the fluorometric method if any of the sixty-three compounds presented auto-fluorescent properties that could interfere with the results obtained in the accumulation assays performed by *Salmonella* NCTC 13349.

When assessed by the semi-automatic fluorometric method using wavelengths of 530/585hp, many compounds presented no auto-fluorescent characteristics, since during the course of the assay they maintained a relative fluorescence of approximately 0%.

In Figure 9 we can observe an example of an auto-fluorescence assay performed for one of the sixty-three hydantoin compounds.

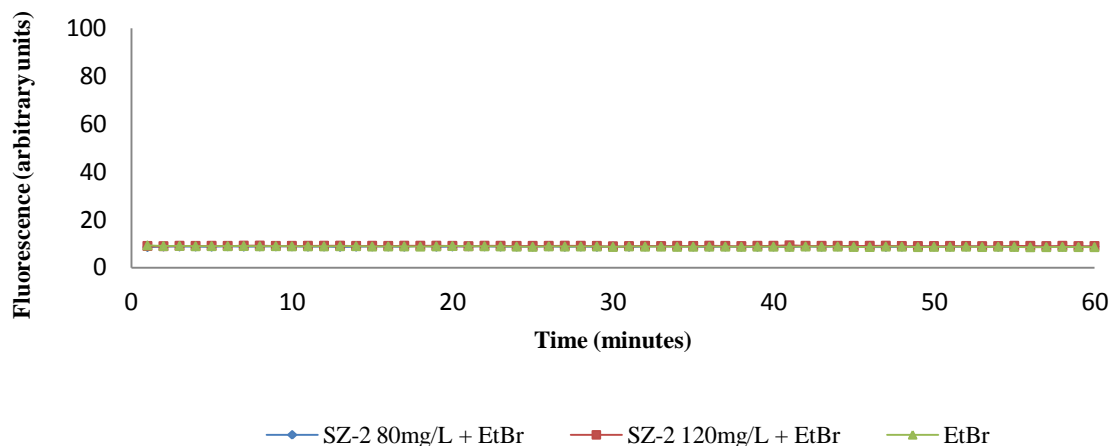


Figure 9 – Auto-fluorescence assay performed for hydantoin compound SZ-2. Example of an auto-fluorescence assay which was performed for all sixty-three compounds at final concentrations of 80 and 120 mg/L, in the presence of PBS at pH 7.4 and EtBr at 1 mg/L.

However, not all compounds were exempt from these auto-fluorescent properties. The hydantoin compounds marked with a * in the Table that follows (Table XIV) presented significant values of auto-fluorescence when the assay was conducted in the presence of EtBr at 1mg/L and PBS at pH 7.4 due to a natural quenching-effect between these hydantoin derivatives and EtBr. When the assay was then conducted in the absence of EtBr, the compounds in question presented no fluorescent properties.

Thus, these compounds were promptly discarded and were not evaluated due to the fact that the auto-fluorescent values obtained were product of the interaction between EtBr and the hydantoin compound under evaluation. Even if these compounds possessed EPIs properties, it was not possible to observe their activity using this semi-automated fluorometric method.

1.2.2. Relative final fluorescence values obtained by the hydantoin derivatives

Because of the extremely large number of graphs depicting the effects of sixty-three hydantoin compounds at two concentrations in an assay that contained a source of metabolic energy (glucose), all results are presented in tabular form. The index of activity of two concentrations of each of the sixty-three hydantoin compounds against the EP system of the *Salmonella enterica* NCTC 13349 was calculated with the aid of the formula described in section 1.2.4.1. of the Material and Methods. Briefly the relative final fluorescence index is a measure of how effective the EPIs inhibitory effect is on efflux (at a certain concentration) by comparison of the final fluorescence (after 60 minutes) of EtBr + EPI exposed cells against cells exposed only to EtBr (absence of EPI).

Table XIV – Relative final fluorescence (RFF) based on accumulation of EtBr. Accumulation of EtBr at 1 mg/L by *Salmonella* NCTC 13349 in the presence of glucose at 0.4% and PBS pH 7.4. The hydantoin derivatives were tested at two different concentrations. The most promising derivative is in bold lettering and underlined.

Agent	Concentration (mg/L)		Agent	Concentration (mg/L)		Agent	Concentration (mg/L)	
	80	120		80	120		80	120
SZ-2	-0,09	0,24	JHP-1	-0,21	0,02	Ksc-7	-0,11	-0,20
<u>SZ-7</u>	<u>3,46</u>	<u>2,62</u>	Fur-2	-0,17	0,04	AD-38	0,01	-0,07
LL-9	-0,20	0,02	GG-2a	0,44	-0,16	AD-39	0,27	0,03
BS-1	0,06	-0,01	GG-4a	-0,05	-0,18	AD-40	-0,01	0,03
JH-63	0,00	0,00	MN-1	-0,44	-0,14	AD-42	0,11	0,13
MN-3	-0,21	0,58	MN-2	0,01	0,02	AD-43	0,15	0,09
TD-7k	0,14	-0,07	MN-4	0,18	-0,19	AD-44	0,21	0,14
GG-5K	-0,54	-0,22	MN-5	-0,02	-0,16	AD-45	0,21	0,48
P3	-0,18	-0,12	MN-6	-0,05	0,10	AD-46	-0,06	-0,02
P7	-0,23	-0,06	BS-5	-0,42	-0,14	AD-49	0,09	0,02
RW-15b	-0,09	0,21	BS-6	-0,16	-0,23	HY-42*	-	-
AD-26	-0,07	-0,13	TN-2	0,04	-0,18	HY-43*	-	-
RW-13	0,14	0,04	TN-3	-0,01	-0,09	HY-44	-0,06	-0,34
AD-29	-0,20	-0,18	BG-1	-0,15	-0,17	HY-46	-0,43	-0,05
KF-2	-0,44	-0,04	BG-2	-0,14	-0,23	HY-47*	-	-
PDPH-3	-0,36	-0,31	JH-1	-0,20	-0,26	HY-72*	-	-
Mor-1	-0,35	-0,10	M-1	-0,19	-0,23	HY-73	-0,14	-0,29

XV	-0,15	-0,23	M-2	-0,11	-0,06	AM-1*	-	-
Thioam-1	1,49	1,83	M-3	0,00	-0,19	AM-2*	-	-
JHF-1	-0,14	-0,36	M-4	-0,22	-0,13	AM-3*	-	-
JHC-2	-0,27	-0,14	Ksc-5	-0,15	0,46	AM-4	-0,05	-0,24

Legend: *compounds that interact with substrate EtBr in the absence of a bacterial strain

As observed in Table XIV, all compounds besides SZ-7 possess low RFF values. Consequently, these compounds were discarded from further evaluation since they most likely do not possess inhibitory activity against *Salmonella enterica* NCTC 13349.

1.2.2.1. Fluorometric assay obtained for hydantoin derivative SZ-7

As evident in Table XIV, no derivative appeared to present activity against the intrinsic EP system of *Salmonella enterica* serovar Enteritidis NCTC 13349 besides compound SZ-7, in the presence of metabolic energy (indicated in Figure 10).

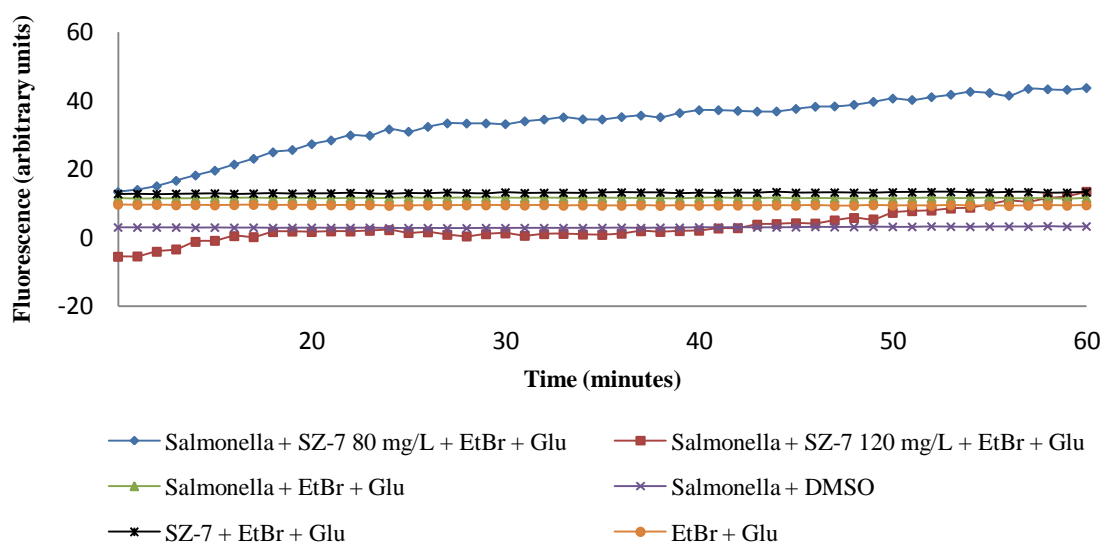


Figure 10 – Accumulation of EtBr by *Salmonella enterica* NCTC 13349 in the presence of varying concentrations of SZ-7. Accumulation of EtBr at 1 mg/L in the presence of glucose at 0.4% and SZ-7 to yield final concentrations of 80 and 120 mg/L. The values represent the accumulation of EtBr by *Salmonella* NCTC subtracted from the fluorescence values obtained in the auto-fluorescence assays, hence the negative values of fluorescence obtained.

As concluded from the data in Figure 10, compound SZ-7 has a valuable inhibitory effect on the EP activity of *Salmonella* NCTC strain and was selected for further experiments to test its ability to inhibit efflux of antibiotics in resistant *Salmonella* mutant strains with over expressed efflux activity.

1.3. MIC values obtained for *Salmonella* mutants 104-CIP and 5408-CIP in the presence of ciprofloxacin, hydantoin compound SZ-7, TZ, and EtBr

Compound SZ-7, which inhibited the efflux of EtBr in reference strain *Salmonella enterica* NCTC 13349, was subsequently employed against two ciprofloxacin-resistant *Salmonella* mutant strains with over-expressed EP activity (77), in the presence of increasing concentrations of ciprofloxacin.

For reasons mentioned earlier, the MIC value for phenothiazine TZ and substrate EtBr was also obtained by *Salmonella* mutant's 104-CIP and 5408-CIP.

Table XV – MIC values obtained for antibiotic ciprofloxacin, hydantoin compound SZ-7, phenothiazine TZ and substrate EtBr by *Salmonella enterica* 104-CIP and 5408-CIP.

Compounds used for MIC determination		<i>Salmonella enterica</i> 104-CIP (mg/L)	<i>Salmonella enterica</i> 5408-CIP (mg/L)
Ciprofloxacin		32	32
SZ-7		> 240	> 240
Ciprofloxacin +	[SZ-7] = 100 mg/L ¹	4	0.469
	[SZ-7] = 50 mg/L ¹	8	8
Ciprofloxacin +	[SZ-7] = 100 mg/L ²	16	0.469
	[SZ-7] = 50 mg/L ²	16	16
TZ		> 256	> 256
Ciprofloxacin +	[TZ] = 100 mg/L ²	8	32
	[TZ] = 50 mg/L ²	32	32

EtBr	> 256	128
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Legend: ¹ Solvent used was DMSO; ² Solvent used was sterile bi-distillated water. [SZ-7]: concentration of SZ-7; [TZ]: concentration of TZ.

As shown in Table XV, we can verify that the association between ciprofloxacin and SZ-7 significantly reduces the MIC value of both ciprofloxacin-resistant strains, when comparing to the MIC value obtained in presence of ciprofloxacin alone and the MIC value obtained for ciprofloxacin in presence of the positive control EPI (TZ).

Given the possibility that the solvent utilized could in some way interfere with the bacterial growth, for this determination both DMSO and double distilled water were used as the solvents for SZ-7. As evident in Table XV, results do reveal that DMSO can slightly inhibit the bacterial growth, when comparing to the results obtained using double distilled water (one dilution difference). However, in both cases there is a decrease in the MIC value, revealing that hydantoin derivative SZ-7 is potential candidate as a bacterial EPI with application for the reversal of efflux based quinolone resistance in *Salmonella*.

1.4. Accumulation of EtBr in the presence of hydantoin derivate SZ-7 in *Salmonella* mutants 104-CIP and 5408-CIP by the fluorometric assay

After observing the synergistic effect between ciprofloxacin and hydantoin derivative SZ-7 on the decrease in the MIC value for ciprofloxacin by *Salmonella* mutant strains with resistance to this class of antibiotics due to the overexpression and activity of EPs (77), we proceeded to assess the behavior of these strains by the semi-automated fluorometric assay previously described.

Ciprofloxacin-resistant strains *Salmonella* 104-CIP and 5408-CIP were submitted to the presence of hydantoin compound SZ-7, in order to verify differences in the accumulation of substrate EtBr, substrate of their over-expressed EPs. Again, fluorescence assays containing phenothiazine TZ were performed in parallel as a positive control, allowing the comparison between compounds.

1.4.1. Effect of hydantoin derivative SZ-7 on accumulation of EtBr in *Salmonella* mutants 104-CIP and 5408-CIP

In Figure 11, we can see that SZ-7 has an inhibitory effect on resistant-ciprofloxacin mutant strains *Salmonella* 104-CIP and 5408-CIP; however there is a noticeable difference of activity between both strains.

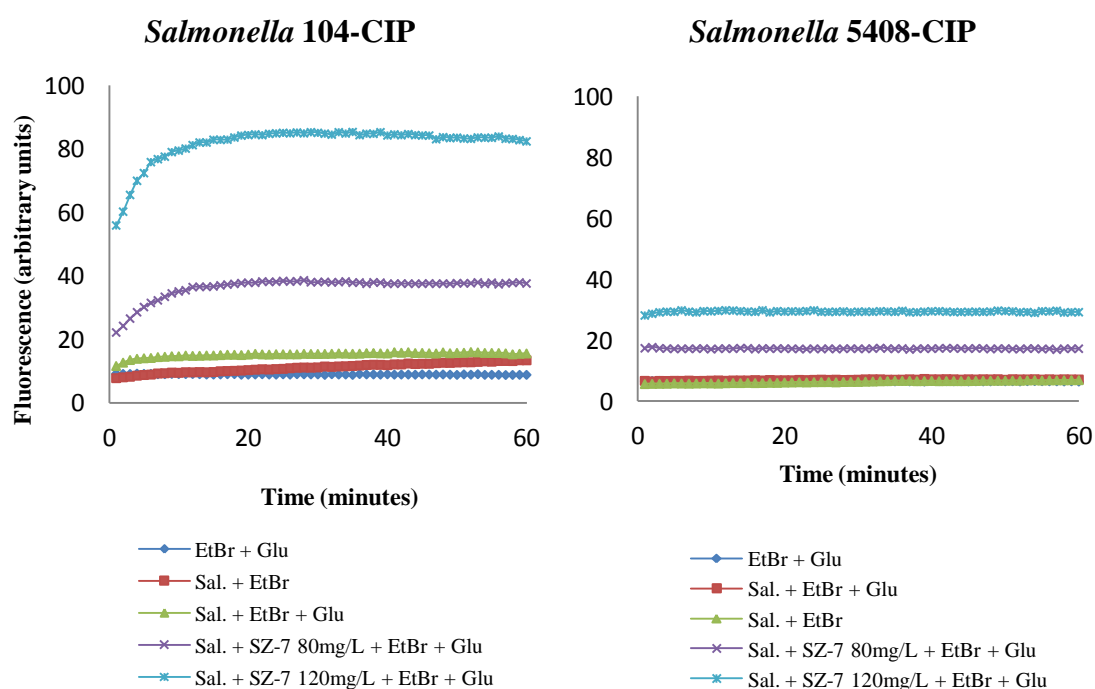


Figure 11 – Accumulation of EtBr by *Salmonella* mutant's 104-CIP and 5408-CIP in the presence of varying concentrations of SZ-7. Accumulation of EtBr at 1 mg/L in the presence of glucose at 0.4% and SZ-7 to yield final concentrations of 80 and 120 mg/L.

Hydantoin compound SZ-7 appears to have a more pronounced effect in the accumulation of EtBr by *Salmonella* 104-CIP, when compared to strain *Salmonella* 5408-CIP.

1.4.2. Effect of TZ on accumulation of EtBr in *Salmonella* mutants 104-CIP and 5408-CIP

As previously pointed out, TZ tests were performed in parallel to serve as a positive control in *Salmonella* mutant strains 104-CIP and 5408-CIP, since phenothiazine TZ is known to have an inhibitory effect against EPs of Gram-negative bacteria (21).

Figure 12 presents the accumulation of EtBr by ciprofloxacin-resistant mutant's *Salmonella* 104-CIP and 5408-CIP, in the presence of sub-inhibitory concentrations of TZ and of a source of metabolic energy.

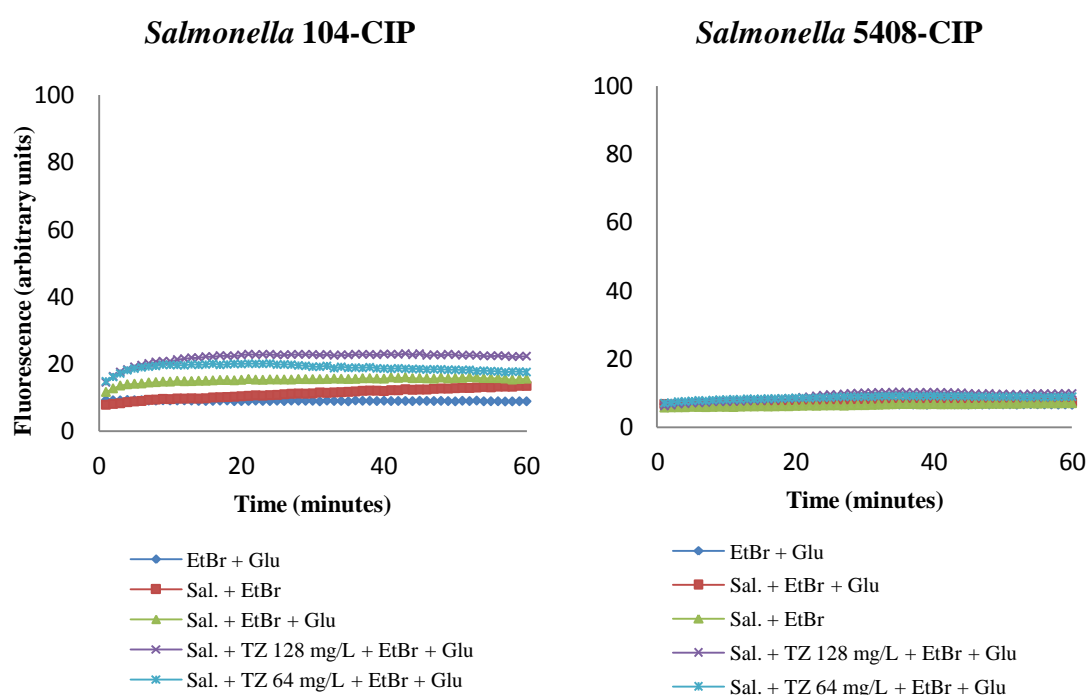


Figure 12 – Accumulation of EtBr by *Salmonella* mutant's 104-CIP and 5408-CIP in the presence of varying concentrations of TZ. Accumulation of EtBr at 1 mg/L in the presence of glucose at 0.4% and TZ to yield final concentrations of 64 and 128 mg/L.

As shown in the graphs above, and compared to the results presented in Figure 8 for reference strain *Salmonella* NCTC 13349, TZ has moderate inhibitory effect on the EP system of both quinolone resistant mutant strains at the concentrations tested. These results put in evidence the significant and specific inhibitory effect that hydantoin

derivative SZ-7 possesses on EtBr efflux of both quinolone resistant mutant strains, in particular *Salmonella* 104-CIP.

Overall these promising results point towards a similar mechanism of action between compound SZ-7 and phenothiazine TZ, with the hydantoin derivative evidencing a much more potent inhibitory effect. Since these derivatives were synthesized in Poland to be tested as putative EPIs for bacterial and cancer cell EPs (46, 98), their scarcity prevented us from further exploring the bioenergetic requirements of the inhibitory effect here revealed by compound SZ-7. Since hydantoins and phenothiazines are related heterocyclic compounds with similar mechanisms of action (20, 52, 98), we decided to further explore this bioenergetic hypothesis using phenothiazines and *E. coli* as the bacterial model where the bioenergetic environment and requirements are best known and elucidated.

2. Assessment of the role of calcium in *E. coli* AG100, in the presence of CPZ and EDTA

Since the CPZ inhibitory effect is known to be related to membrane transport channels dependent upon calcium ions (Ca^{2+}) and ATPases activity, the role of Ca^{2+} as well as pH was investigated in order to clarify the affect of these ions on active efflux which consequently interferes with the activity of EPIs, in particular the calcium channel inhibitors. Previous results obtained by our research group concluded that at pH 5 efflux is independent of metabolic energy; however, at pH 8 it is entirely dependent of metabolic energy and Ca^{2+} is essential to maintain the activity of the bacterial ATPases (5, 52).

2.1. MIC values obtained for *E. coli* AG100 in the presence of CPZ, EDTA and EtBr

Phenothiazines such as CPZ and thioridazine (TZ) inhibit the binding of Ca^{2+} to enzymes involved in supplying energy from the hydrolysis of ATP (2). Therefore, Ca^{2+} plays a crucial role in cellular signaling, biochemical pathways, membrane transport channels and in the activity of certain ATPases (67, 72).

To be able to evaluate these effects in the semi-automated fluorometric assay, we initially determined the MIC values for EPI CPZ, Ca^{2+} chelator EDTA and substrate EtBr. The results obtained will provide information as to what concentrations do not affect cellular viability and replication (108). The assays were repeated three times, in order to guarantee reproducibility of the results obtained.

Table XVI – MIC values obtained by *E. coli* AG100 in the presence of EPI CPZ, Ca^{2+} chelator EDTA and substrate EtBr.

<i>E. coli</i> K-12	MIC for CPZ (mg/L)	MIC for EDTA (mM)	MIC for EtBr (mg/L)
AG100	80	2.5	150

2.2. Accumulation of EtBr in the presence of CPZ and EDTA to assess the role of calcium in *E. coli* AG100 strain by the fluorometric assay

Since these phenothiazines have major effects on the accumulation and efflux of EtBr at pH 7 (107), and because these effects can be modified by metabolic energy, the role of Ca^{2+} in the modulation of EtBr accumulation and efflux was evaluated.

Because CPZ has a greater outcome on accumulation of EtBr by *E. coli* AG100 strain than other phenothiazines, CPZ was chosen in order to evaluate the role of Ca^{2+} . In addition, due to the chelating properties of EDTA and its ability to ‘sequester’ metal ions such as Ca^{2+} and Fe^{3+} , this agent was also used to study the role of Ca^{2+} on the efflux of EtBr.

As pointed out previously, this technique provides the ability to continuously monitor the transport of substrate EtBr, making it the ideal tool to evaluate accumulation/efflux by EP systems (74, 107), when in the presence of diverse physiological conditions.

2.2.1. Effect of CPZ on accumulation of EtBr and modulation of that effect by Ca^{2+} ions

2.2.1.1. Effect of CPZ at varying concentrations on the accumulation of EtBr

As expected, Figure 13 confirms that increasing concentrations of calcium channel inhibitor CPZ promotes analogous increases in the amount of relative fluorescence, which is associated with increased accumulation of EtBr by *E. coli* AG100 at pH 8, as result of the EP inhibition (107).

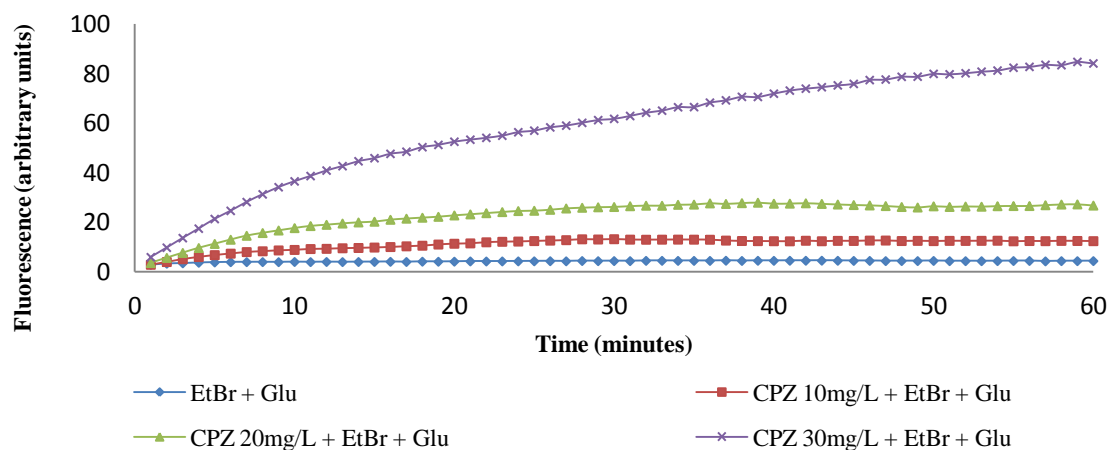


Figure 13 – Effect of chlorpromazine (CPZ) on accumulation of EtBr. EtBr accumulation by *E. coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4% and CPZ to yield final concentrations of 10, 20 and 30 mg/L.

2.2.1.2. Ca^{2+} modulation of the effects of CPZ on accumulation of EtBr

Due to the ability that CPZ has on inhibiting various EP systems by inhibiting the transport of Ca^{2+} (56), the potential modulating effect of Ca^{2+} on the activity of CPZ was investigated.

As seen in Figure 14, the activity of CPZ on the accumulation of EtBr can be completely abolished by the addition of 5mM of Ca^{2+} to the assay.

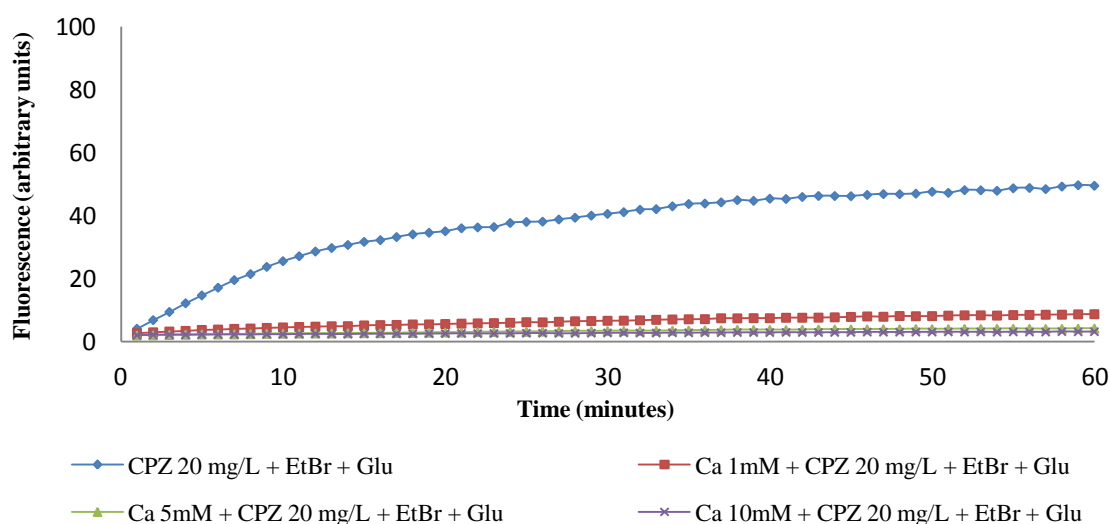


Figure 14 – Modulation of the effect of CPZ by calcium ions (Ca^{2+}). EtBr accumulation by *E. coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4%, CPZ at 20 mg/L and calcium chloride to yield final concentrations of 1, 5 and 10 mM.

This result supports our hypothesis that Ca^{2+} plays a key role in the mechanism by which EP systems function in Gram-negative bacteria and strongly modulates the activity of Ca^{2+} channel inhibitors.

2.2.2. Effect of EDTA on accumulation of EtBr and modulation of that effect by Ca^{2+} ions

2.2.2.1. Effect of EDTA at varying concentrations on the accumulation of EtBr

To confirm this data, we proceeded in determining if the addition of Ca^{2+} chelator EDTA would present similar outcomes when in the presence of Ca^{2+} . As seen in Figure 15, the addition of EDTA to the assay promotes a modest accumulation of EtBr, owing its ability to retain ions such as Ca^{2+} .

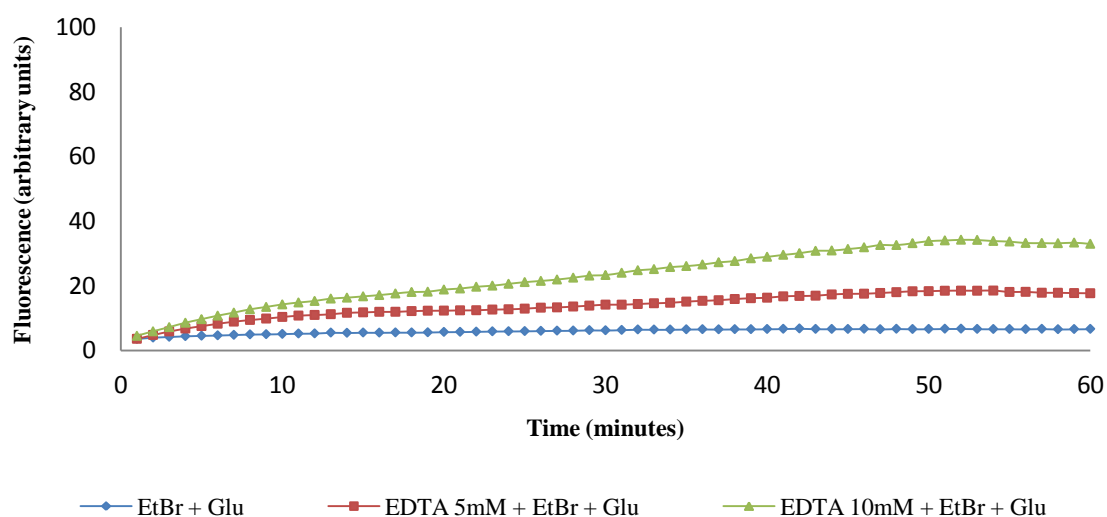


Figure 15 – Effect of EDTA on the accumulation of EtBr. EtBr accumulation by *E. coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4% and EDTA to yield final concentrations of 5 and 10 mM.

2.2.2.2. Ca^{2+} modulation of the effects of EDTA on accumulation of EtBr

Afterwards, we evaluated if these cumulative effects induced by EDTA could be reversed by the presence of Ca^{2+} . As shown in Figure 16, Ca^{2+} modulates the effects of varying concentrations of EDTA, which is demonstrated by the accentuated decrease in

accumulation of EtBr. Therefore, the activity of EDTA on the accumulation of substrate EtBr can also be abolished by the addition of Ca^{2+} ions to the assay.

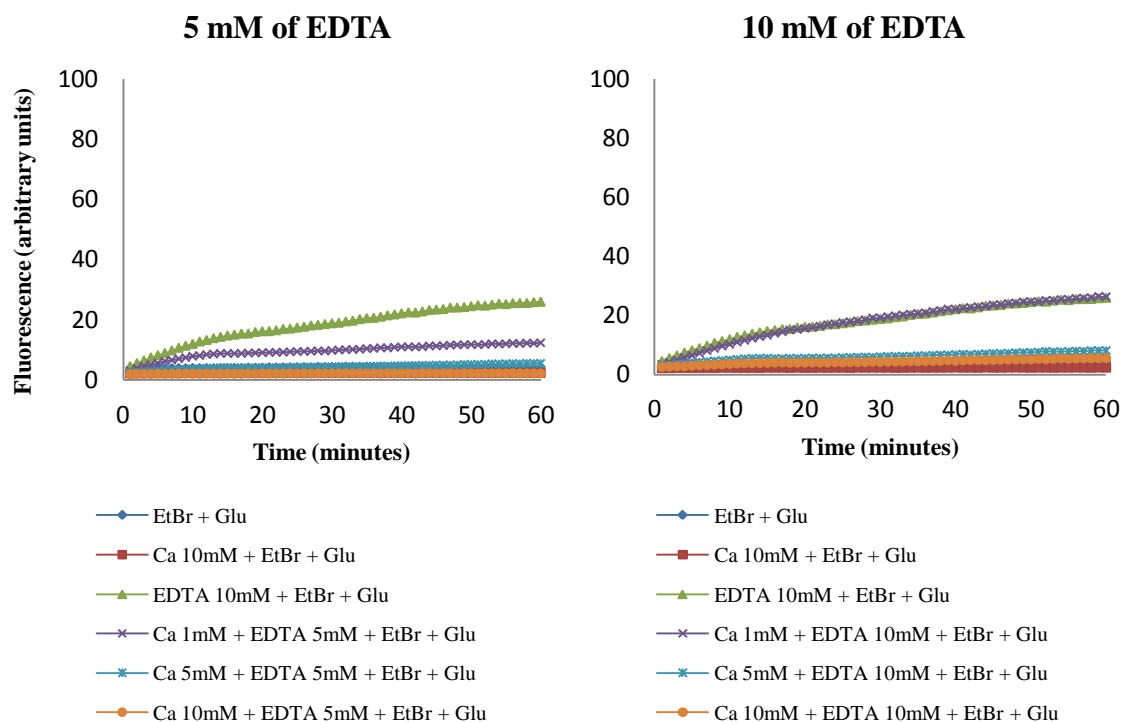


Figure 16 – Modulation of the effect of 5 and 10 mM of EDTA by Ca^{2+} ions. EtBr accumulation by *E. coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4%, EDTA at 0.0, 5 and 10 mM, and calcium chloride to yield final concentrations of 0.0, 1, 5 and 10 mM.

2.2.3. Effects of CPZ with and without Ca^{++} on efflux of EtBr

To provide data that indeed it is the EP system that is affected by the presence of EPI CPZ, an efflux assay was performed, as shown below in Figure 17.

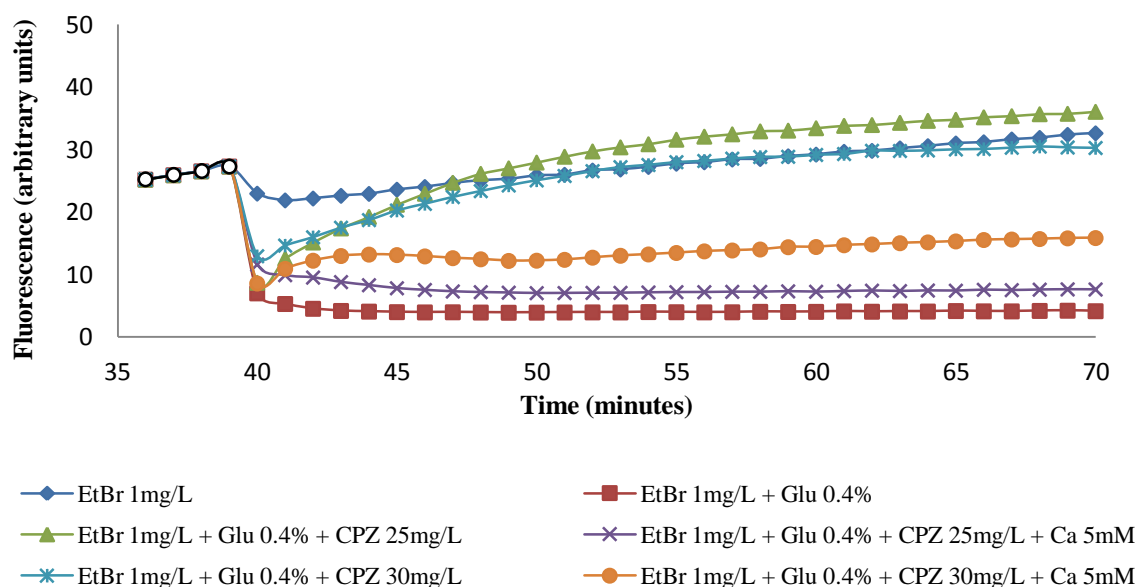


Figure 17 – Effects of chlorpromazine (CPZ) with and without Ca^{2+} on efflux of EtBr by *E. coli* AG100 strain.

At the beginning of the assay, all tubes accumulated EtBr at 1 mg/L and PBS at pH 8. The addition of glucose at 0.4% and CPZ at 25 and 30 mg/L took place at approximately 40 minutes, with and without 5 mM calcium chloride.

The first 35 minutes of the assay which are not presented in Figure 17 served for the sole purpose to allow the accumulation of EtBr in the absence of glucose to take place. However, as shown in Figure 17, subsequent efflux of EtBr can be strongly modulated by the addition of glucose, which accounts for complete efflux for the duration of the assay. When glucose and CPZ at two concentrations that do not affect replication of the organism are included in the assay, each one inhibits efflux of EtBr in a concentration dependent manner. The addition of glucose and Ca^{2+} to the tubes containing CPZ induces the abolishment of the inhibitory effect of CPZ on efflux of EtBr.

V. DISCUSSION AND CONCLUSION

Bacteria have evolved sophisticated mechanisms of resistance including efficient drug EPs that accommodate a wide range of substrates, both antibacterial and non-antibacterial. Thus, it has become indispensable to optimize the pharmacokinetics and pharmacodynamics of antibacterial therapy (42) and develop ideal EPIs that assist antibacterial agents in combination therapy.

The design of effective and selective EPIs requires an extensive screening process that involves various compounds, whether they have natural or synthetic origin. With respect to hydantoin derivatives, over the past several years researchers have conducted studies that explore various aspects of their biochemical and pharmacological properties, since these compounds present beneficial physicochemical properties in the field of medicine.

For this particular study, one of the primary objectives focused on assessing possible inhibitory effects inherent to sixty-three hydantoin derivatives synthesized in the Department of Technology and Biotechnology of Drugs, Medical College, Jagiellonian University, Cracow, Poland by Dr. Jadwiga Handzlik and Prof. Dr. Katarzyna Kiec-Kononowicz, on the intrinsic efflux activity of *Salmonella enterica* serovar Enteritidis NCTC 13349. Then, after the screening process, we subjected the most promising hydantoin derivatives to the presence of an antimicrobial agent and evaluated their behavior, but this time employing ciprofloxacin-resistant mutant strains *Salmonella enterica* 104-CIP and 5408-CIP, two mutant strains with increased expression and activity of EPs responsible for a quinolone resistant phenotype.

In order to do so, two experimental techniques were applied to explore these inhibitory properties: a) determination of minimal inhibitory concentrations (MIC) by the microdilution method in presence and absence of the modulator and antibiotic, and b) a semi-automated real-time fluorometric method, which employs EP substrate EtBr. The MIC determinations for all sixty-three compounds in the presence of *Salmonella enterica* serovar Enteritidis NCTC 13349 provided information as to what concentration of the compound under study was capable of inhibiting bacterial growth and affecting

cellular viability. From this assay we observed that none of the compounds were able to inhibit the growth of *Salmonella enterica* serovar Enteritidis NCTC 13349 at concentrations as high as 240 mg/L. As for the control EPI, TZ was also unable to inhibit bacterial growth at concentrations as high as 256 mg/L.

After these determinations, we proceeded in evaluating all sixty-three hydantoin compounds by the semi-automated fluorometric method. This method offers the possibility to screen a great number of potential EPIs providing information about the transport kinetics that take place, in a time-saving, accurate and inexpensive way. By detecting the balance between accumulation and efflux of EtBr in a real time basis, it provides the possibility to alter certain parameters like temperature and pH at which the assay will occur.

However, other parameters must remain unaffected, since they are essential for the proper functioning of the assay and, consequently, in obtaining reliable results. These requirements are: i) the concentration of EtBr free in the extracellular environment, the concentration of all compounds tested and their solvents must not affect cellular viability; ii) the compound and/or its solvent should not interfere with the fluorescence signal in the thermocycler; iii) cells should be given the ideal conditions in terms of their energy source (glucose at 0.4%), while testing potential efflux inhibitors. This ensures that in optimal energy conditions, the inhibitory effect that may be produced on efflux is most likely due to the EPI under evaluation.

Therefore, this study progressed with the assessment of possible auto-fluorescent properties of all hydantoin compounds undergoing evaluation, in the presence of EtBr that could interfere with the subsequent evaluation of their inhibitory effects against the intrinsic EP systems of *Salmonella enterica* serovar Enteritidis NCTC 13349. All compounds that presented these properties were promptly discarded from the study since it would not be possible to evaluate their inhibitory behavior using this technique.

In order to respect the parameters set forth above, all compounds were utilized at half and at a quarter of their MIC values, in order to guarantee bacterial viability and the capacity to extrude any compound to which they were subjected.

EtBr was used at the lowest concentration in which accumulation was observed by *Salmonella* NCTC 13349, to guarantee the minimum fluorescent level representing the basal steady-state level between accumulation and efflux of EtBr (steady-state reached around 10% of relative fluorescence from the instrument). Thus, when the strain is subjected to the presence of hydantoin derivatives and/or antimicrobial agents and the abrupt accumulation of EtBr occurs due to efflux inhibition, EtBr accumulates in the periplasm and the increased fluorescence is possible to monitor since the fluorescence values obtained are within the range of readings of the instrument. Otherwise, if the concentration of EtBr used was too high, when subjecting the strain to the inhibitory effect of hydantoin compounds and quinolones, it would not be possible to monitor that activity, since the fluorescence values obtained would cross over the reading limit of device.

In this assay, the selection of the pH of the medium was approximated to the pH value present in interstitial fluids, namely, a pH of 7.4. This way, the *in vitro* activity of the agent against the EP system of a given bacterium provides meaningful results possible to correlate to *in vivo* activity. For this reason, the assays that tested all sixty-three hydantoin derivatives by the fluorometry technique were conducted at pH 7.4, in the presence of glucose (0.4%) as the source of metabolic energy.

As for the results obtained, with the exception of compound SZ-7, none of the other hydantoin derivatives presented inhibitory activity against the EP system of *Salmonella enterica* serovar Enteritidis NCTC 13349. The only compound capable of enhancing EtBr accumulation was derivative SZ-7, in the presence of optimal efflux conditions, i.e., in the presence of glucose and a pH of 7.4.

Given the results obtained in the semi-automated fluorometric assay, we re-assessed compound SZ-7, in order to demonstrate its effectiveness. With this intent, we used induced ciprofloxacin-resistant mutants *Salmonella enterica* serovar Enteritidis 104-CIP and 5408-CIP with a quinolone resistant phenotype due to increased expression and activity of EPs.

Initially, for both mutant strains the MIC values were determined for derivative SZ-7 and for ciprofloxacin. Taking into account the values obtained, a further determination

was performed by adding a single concentration of SZ-7 to the wells containing increasing concentrations of ciprofloxacin. This association allowed us to observe a significant decrease of the MIC value in the presence of both ciprofloxacin and SZ-7, when comparing with the MIC value obtained by ciprofloxacin and SZ-7 separately, and also by the results obtained in the control assay that employed TZ. A clear synergistic effect between ciprofloxacin and SZ-7 was demonstrated, with reversal of resistance of the mutant *Salmonella* strains.

When we proceeded in evaluating the inhibitory effect of SZ-7 in both *Salmonella* mutant strains by the fluorometric assay we verified that the hydantoin derivative presented indeed the capacity to inhibit the efflux of substrate EtBr, possibly by inhibiting the activity of over-expressed EPs. However, it is important to stress the differential inhibitory effect of SZ-7 against both mutant strains. According to O'Regan et al (77), the parent *Salmonella* Enteritidis strains 104 and 5408 displayed high-level nalidixic acid resistance, reduced susceptibility to ciprofloxacin and harbored a single GyrA mutation (D87Y). After the exposure to ciprofloxacin, both mutant strains were genotypically characterized. Mutant 104-CIP harbored two GyrA (D87Y and S83F) mutations, and 5408-CIP harbored single GyrA (D87Y), GyrB (E466D), and ParE (V461G) mutations. Both mutants over-expressed *acrB*. The global regulator genes *soxS* and *marA* were over-expressed in 104-CIP, and *ramA* was over-expressed in 5408-CIP. Mutations were found in SoxR (R20H) and in SoxS (E52K) in 104-CIP and in RamR (G25A) in 5408-CIP.

Thus, even though both strains possess an over-expression of the *acrB* gene, mutational differences can influence the inhibitory effect of hydantoin derivative SZ-7. Despite the differences observed, in both strains it was possible to note the inhibitory effect of derivative SZ-7 in the efflux of EtBr, thus allowing us to conclude that hydantoin compound SZ-7 has a direct or indirect effect against the activity of the AcrAB efflux system.

The fact that both ciprofloxacin and SZ-7 reduced the MIC value provided an indication that this compound possesses a physical-chemical propriety that is worth further study,

especially against EPs of cancer cells (98), and revealed that the hydantoin derivative is a potential candidate for bacterial EPI with application for the reversal of EP based quinolone resistance in *Salmonella* strains.

The study of structural analysis and biochemical activity of derivative SZ-7 would enable a better understanding and interpretation of the results, apart from the fact that it would also provided information regarding the mechanisms inherent to the compound that are capable of triggering this inhibitory effect. Since these derivatives were specifically synthesized in Poland to be tested as putative EPIs for bacterial and cancer cell EPs, their scarcity prevented us from further exploring these hypothesis and the remaining SZ-7 was conveyed towards the evaluation of its activity against EPs of cancer cells (98). Structural and molecular based activity studies, more specifically quantitative structure-activity relationship analysis (QSARS), will be carried out in the future after re-synthesizing more compound and patent rights have been assigned by the Polish collaborator research group.

After evaluating the newly synthesized hydantoin compounds, we redirected the present MSc study towards the research of aspects inherent to the functioning of bacterial cells that can influence the screening process of potential EPIs, taking advantage of the fact that hydantoins and phenothiazines are heterocyclic compounds (9, 20) with putative similar mechanisms of action regarding the neurological system (20, 76). To explore the bioenergetic hypothesis of this thesis we have continued the research work using phenothiazines and *E. coli* as the bacterial strain. Hence, we devoted the second part of the present study towards the bioenergetic machinery and biochemical requirements behind the activation of efflux systems in bacterial cells.

Having said that, we questioned whether Ca^{2+} ions would have a decisive influence in the regulation of the EP systems present in reference strain *E. coli* AG100, and how this regulatory function could affect potential EPIs.

In bacterial cells, Ca^{2+} is implicated in a wide variety of cellular processes, including the cell cycle, cell division (60) and many other mechanisms mentioned earlier.

On the other hand, CPZ is recognized for its inhibitory properties on the activity of EP systems by restraining the binding of Ca^{2+} to energy providing enzymes (4, 57). *In vitro*, CPZ is capable of promoting the accumulation of bacterial EP substrate EtBr that results from the inhibition of bacterial efflux (54). However, previous studies demonstrated that the effects of a phenothiazine as efflux inhibitors are not evident at pH 5, regardless of whether metabolic energy is present (52). Owing the reason previously stated, in this study it was hypothesized that all assays are performed using PBS at a pH 8 and always in the presence of glucose as the immediate source of metabolic energy. This requisite reflects conditions at the surface of the cell where hydronium ions resulting from metabolic activity are stored, and due to the dissociation of these ions into the bulk medium at pH 8 they must be restored by glucose via metabolic activity (52).

These requirements allow the bacterial strain to perform in optimal conditions since maintenance of the proton motive force (PMF) is favored, consequently promoting efflux activity.

In the beginning of this study, accumulation of bacterial EP substrate EtBr is confirmed in the presence of varying concentrations of CPZ, which resulted in the inhibition of efflux. However, when calcium chloride is added to the assay in the presence of CPZ, an abrupt decrease in accumulation of EtBr occurs. Because the inhibitory effects of CPZ on efflux of EtBr can be abolished by the addition of Ca^{2+} , the role of calcium in this assay is proven to be being essential for the maintenance of efflux by reference strain *E. coli* AG100.

The effects of calcium ions were later on confirmed by utilizing Ca^{2+} chelator EDTA as the inhibitor of efflux activity. As is the case for CPZ, varying concentrations of EDTA present inhibitory effects on efflux of EtBr, although not as evident as with CPZ. For this assay it was necessary to use higher concentrations of EDTA, when comparing to the MIC values obtained for this compound. Even so, cellular viability is still guaranteed owing the fact that for the duration of the assay (approximately 60 minutes) the concentrations employed are not capable of affecting the viability of *E. coli* AG100, contrary to the 18 hour incubation period of a MIC determination. Nevertheless, the inhibitory effects that EDTA was able to provide in the assays are also completely obviated by the addition of Ca^{2+} ions, allowing the cell to evade the inhibition of efflux.

Because the inhibitory effects of CPZ on efflux of EtBr can be completely precluded by the addition of Ca^{2+} ions, we conclude that the effects of CPZ on EP systems of reference strain *E. coli* AG100 are entirely reversible via the bioenergetic and biochemical requirements for active efflux.

CPZ is only capable of inhibiting efflux activity when the bacterial medium lacks the pool of ions it needs to support bacterial growth and replication. Furthermore, we consider that when *E. coli* AG100 is found in standard conditions of growth and replication, Ca^{2+} ions that are re-utilized to bind to calcium-dependent enzymes for the survival of bacterial strains are inhibited by CPZ, leaving no more available for Ca^{2+} -dependent enzymes responsible for providing energy to the bacterial strain.

Also, at pH 8 CPZ is capable of demonstrating a strong inhibitory activity on the efflux system of reference strain *E. coli* AG100. This system is primarily maintained due to the presence of glucose as the immediate energy source. Hence, by performing the assays at this pH, we confirm that the inhibitory activity results from the direct inhibition of energy to *E. coli*, i.e., the inhibition conducted by CPZ acts directly on the energy supplying enzymes (bacterial ATPases), an enzymatic process that is dependent upon Ca^{2+} , and not by a direct influence on the EPs systems themselves.

The results acquired above provide data that Ca^{2+} plays a key role in the efflux mechanism of *E. coli* reference strain AG100. To complement and confirm this data an additional assay was performed by Ana Martins *et al* (54), which questioned whether the initial effects of CPZ on the EP system of *E. coli* AG100 are in fact reversible upon the addition of Ca^{2+} ions. In the efflux assay performed (54), results confirm that the addition of Ca^{2+} restores the cells efflux capacity which had been reduced in the accumulation phase by the presence of CPZ alone. However, when the assay was conducted in the presence of both CPZ and EDTA, the accumulation is much more significant and the addition of the same concentration of Ca^{2+} is no longer adequate to reverse the inhibitory effects produced.

Therefore, taking into consideration all the data gathered during this study, it is safe to say that Ca^{2+} does in fact possess the capacity to completely obviate and reverse the effects of phenothiazine CPZ, a requisite that is not achievable by the addition of

glucose as the immediate energy source. Metabolic energy which is provided to *E. coli* AG100 is not solely responsible for energizing the EPs. This system depends on a large range of variables, many of which possibly remain unknown.

In conclusion, this study allowed us to verify that there is a much more complex mechanism inherent to all bacterial strains involving the regulation and activation of EP systems and its relation with antibiotic resistance. While this mechanism remains unknown, the screening and selection of new and potent compounds, like hydantoin derivative SZ-7, that might provide the ideal properties and characteristics to become excellent EPIs remains a difficult task to accomplish. Overall this work puts in evidence that many biochemical and bioenergetic aspects related to the strains physiology need to be taken into consideration in bacterial drug resistance mediated by EPs.

Future Perspectives

Both gram-positive and gram-negative bacteria resistant to most antibacterial classes available have become increasingly prevalent as nosocomial pathogens, particularly among immune-compromised and hospitalized patients. Many of these pathogens become multi-drug resistant by the increased expression and activity of EPs capable of extruding antiseptics, disinfectants and antibiotics. This fact highlights the importance and the emergency needed for the development of alternative antimicrobial therapies to prevent the growth and expansion of these MDR bacterial strains worldwide. The search for new and potent EPIs is much needed as well as the unraveling of the biochemical and bioenergetic requirements behind active efflux, in order to effectively design efficient combinational/synergistic therapies with antibiotics and EPIs to prevent the emergence of these strains.

Regarding the importance of hydantoin derivatives, these compounds are known to possess non-toxic properties and if derivative SZ-7 is shown to be non-toxic (on-going work in eukaryotic cells), evaluating its potential to cure mice with an efflux mediated MDR *Salmonella* infection would be worthwhile. Also, the application of quantitative structure-activity relationship analysis should be applied in order to correlate the biological activity of the compound with a wide variety of physical and chemical parameters.

Regarding the importance of ions (i.e., Ca^{2+}) which participate in the complex network that is EPs, a better understanding of the metabolic processes involved in the molecular basis of efflux transport and inhibition is absolutely necessary to progress in the drug development field. This knowledge will make it possible to improve the screening process of compounds with potential to behave as inhibitors of EPs.

The development of non-toxic and specific inhibitors of EPs, particularly those that interfere with specific bacterial energy requirements, will facilitate the chemotherapeutic antibacterial regimens, not only preventing multi-drug resistance but also enhancing the efficacy of normal therapeutic doses against drug resistant bacteria and thus allow the reuse of existing chemotherapeutic arsenal contributing to combat the scourge of antibiotic resistance in bacteria.

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